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The Uses and Properties of PEG-Linked Proteins

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ABSTRACT: Poly(ethylene glycol) (PEG) is a water soluble polymer that when covalently linked to proteins, alters their properties in ways that extend their potential uses. PEG-modified conjugates are being exploited in many different fields. The improved pharmacological performance of PEG-proteins when compared with their unmodified counterparts prompted the development of this type of conjugate as a therapeutic agent. Enzyme deficiencies for which therapy with the native enzyme was inefficient (due to rapid clearance and/or immunological reactions) can now be treated with equivalent PEG-enzymes. PEG-adenosine deaminase has already obtained FDA approval. PEG-modified cytokines have been constructed and, interestingly, one of the conjugates, PEG-modified granulocyte-macrophage colony-stimulating factor, showed dissociation of two biological properties. This novel observation may open new horizons to the application of PEGylation technology. The biotechnology industry has also found PEG-proteins very useful because PEG-enzymes can act as catalysts in organic solvents, thereby opening the possibility of producing desired stereoisomers, as opposed to the racemic mixture usually obtained in classical organic synthesis. Covalent attachment of PEG to proteins requires activation of the hydroxyl terminal group of the polymer with a suitable leaving group that can be displaced by nucleophilic attack of the ϵ-amino terminal of lysine residues (other nucleophilic groups can also interact). Several chemical groups have been exploited to activate PEG, thereby giving rise to a variety of PEG-proteins. Some of these varieties retain part of the activating group as a coupling moiety between PEG and protein and others provide a direct linkage. For each particular application, different coupling methods provide distinct advantages. The development of newer methods should increase the number of biologically active PEG-proteins and, possibly, the number of applications. Also, a deeper understanding of the molecular basis for the altered physicochemical properties will facilitate the development of designed PEG-modified proteins with the desired spectrum of biological activities and, hence, new applications.

KEY WORDS: poly(ethylene glycol), therapeutic use, biotechnological processes, analytical techniques, coupling methods, proteins, cytokines, enzymes.

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I. INTRODUCTION

Chemical modification of proteins has been extensively used to alter the properties of the protein and to gain information on structural features. One of the objectives of the chemical modification is to produce proteins which can be used as therapeutic agents. Specific protein domains can be "hidden" by the modifier in order to increase the plasma half-life of the protein, reduce immunogenicity and antigenicity, and increase resistance to proteolysis. A second objective is to alter physicochemical properties. These new modified proteins can be used in environments unsuitable for the native protein, e.g., enzymic catalysis in organic solvents. A variety of chemical modifiers has been used to achieve each of these two objectives. Poly(ethylene glycol) (PEG) is a versatile polymer having hydrophylic and hydrophobic properties that have been successfully used to improve the pharmacological properties of proteins as well as to produce enzyme conjugates for bioreactors.

This article reviews the "state-of-the-art", focusing on the implications of the observations rather than giving an exhaustive description of the numerous papers published in this field. Section II describes the benefits of PEG modification for protein pharmaceuticals, followed by the applications of PEG-proteins for biotechnological purposes. Details of the PEG coupling methods in Section IV are followed by a description of the analytical and preparative problems of PEG-proteins.

II. PEG-PROTEIN PHARMACEUTICALS

The advent of recombinant DNA technology has brought with it the rapid development of protein therapeutics. Cytokines and other biological response modifiers, thrombolytics, adhesion molecules, and agonist and antagonist peptide fragments of growth factors and their receptors all have widespread applications.[1] Enzymes of bacterial origin also have potential in replacement therapies. The full exploitation of this new and powerful therapeutic armory requires solutions to several problems. First, most parenterally administered proteins are rapidly cleared from the circulation by the reticuloendothelial system (RES), kidney, spleen, or liver. Clearance depends on size (relative to the cutoff for glomerular filtration), charge, attached carbohydrate chains, and the presence of cellular receptors for the protein. Metabolism by proteases/peptidases also leads to the rapid loss of biological activity and degradation. Second, proteins of bacterial origin are immunogenic, and even with recombinant human proteins immune responses are elicited after repeated use. Such antibodies can neutralize biological activity (if they bind to key epitopes), cause life-threatening hypersensitivity reactions, and increase clearance by the RES.

Sustained release technologies (liposomes and microspheres) have made some progress, although both suffer from the problems of being rapidly sequestered in the liver and immunological responses (they can act as immunological adjuvants).[2]

Of the chemical modification technologies explored to date, PEG-modification appears to be the most promising. Over 40 proteins have now been studied and a clear pattern has emerged. PEG-modification almost invariably achieves lengthened plasma half-life, reduced antigenicity, and reduced sensitivity to proteolysis.[3] The pharmacological benefits of PEG-modification also extends to other compounds. PEG-adriamycin has a high anticancer activity and reduced toxicity when compared with unmodified adriamycin.[4] Some of the problems posed by liposomes are also alleviated by PEG-modification.[5–9] Thus, this seems to be the technology with which to increase the pharmacological usefulness not only of the drugs but also of the carriers. Given the good toxicology record of PEG, it has a clear advantage over other chemical modifications.

A. Generic Benefits of PEG-Modification of Proteins for Clinical Use

1. Increased Plasma Half-Life

PEG-modification has extended the plasma half-life of numerous proteins by a factor of 3 to 486-fold (Tables 1 and 2). Several mechanisms account for this, including the increased size of proteins normally below the limit for glomerular filtration, interference with the interaction of carbohydrate chains with their specific receptors, masking specific aminoacid sequences for which there are cellular receptors, and reduced proteolysis and antigenicity. The extent of improvement in plasma half-life is not clearly related to protein size, charge, or type. It may be significant, however, that albumin, a protein intended to be retained in the circulation, does not show a significantly improved half-life with PEG-modification (Table 2).

In general, the more PEG attached per protein molecule the greater the extension of half-life,[10–13] but the price paid may be the reduced specific activity of the protein (see below). Where proteins show biphasic clearance, both α and β components are increased (cf. Interleukin-2, Table 2). For some proteins, only

TABLE 1
Influence of PEG-Modification on Proteins Plasma Half-Life in Man

Protein	Half-life (h)		Fold increase	Ref.
	Native protein	PEG-protein		
Asparaginase	20	357	17.7	204
Asparaginase	72	528	7.3	71
Glutaminase-asparaginase	<0.5	72	>144	205
Uricase	<3	8	>2.7	58
Superoxide dismutase	0.42	204	486	68

TABLE 2
Influence of PEG-Modification on Proteins Plasma Half-Life in Other Species

Protein	Animal	Half-life (h)		Fold increase	Ref.
		Native protein	PEG-protein		
Asparaginase	Mouse	<6	96	>16	206
Asparaginase	Rat	2.9	56	19.3	60
Asparaginase	Rabbit	20	144	7.2	70
Glutaminase-asparaginase	Mouse	2	24	12	73
Superoxide dismutase	Mouse	0.06	17	283	12
Lactoferrin (PEG-2000)	Mouse	0.05	0.25	5	12
Lactoferrin (PEG-4000)	Mouse	0.05	1	20	12
Streptokinase	Mouse	0.07	0.33	4.7	13
Plasmin-streptokinase complex	Mouse	0.05	0.22	4.4	13
Adenosine deaminase	Mouse	0.5	28	56	207
Interleukin-2	Rat	α0.05	0.32	6.4	15
		β0.73	6.8	9.3	
Bovine albumin	Rabbit	β93	96	1.03	35

the PEG-modified form is cleared biphasically,[12] presumably reflecting retention of the material for a sufficiently long period, thereby allowing a second, slower, clearance mechanism to make a significant contribution. PEG-SOD has been shown to bind to blood cells via interaction of the PEG-chains with the cell membranes,[14] and this might account for some of the improvement in half-life.

a. Reduced Renal Clearance

The cutoff point for glomerular filtration lies between albumin (molecular mass 67 kDa), which is retained, and hemoglobin (molecular mass 68 kDa), which is filtered (with some variation in the filtration of circa 70 kDa proteins due to shape, charge, etc.). Plasma clearance of unmodified rIL-2 is essentially that predicted for a small protein cleared by glomerular filtration.[15] Experiments with nephrectomized animals[16] support this. The decrease in systemic clearance rate (and associated increase in plasma half-life) of PEG-rIL-2 is not marked when the effective molecular size increases from 19.5 to 21 kDa but occurs largely between 66 and 70 kDa.[15] It is thus likely to be due largely to exclusion of the protein from glomerular filtration. However, because above the molecular size of 70 kDa the systemic clearance of PEG-rIL-2 does not drop to zero and it is further reduced by addition of more PEG, an additional clearance mechanism is anticipated.[15] Knauff et al.[15] demonstrated degradation products in the urine with both rIL-2 and PEG-rIL-2. Degradation prior to filtration is, however, unlikely given the abrupt change in clearance as the 70-kDa threshold is exceeded,

suggesting metabolism by cells lining the proximal tubule or other sites in the kidney.

Experience with hemoglobin modified with PEG-4000 and PEG-1900 is similar and consistent with reduced renal clearance.[10] Superoxide dismutase is also cleared by the kidney and shows a dramatic increase in half-life when modified (Tables 1 and 2), which is much more marked than that for interleukin-2.

b. Reduced Cellular Clearance

Systemically administered proteins of over 70 kDa are rapidly cleared by the RES or by specific cell/protein interactions. PEG appears to reduce cellular clearance, irrespective of the mechanism. For example, lactoferrin is cleared by interaction with carbohydrate receptors on hepatocytes.[12] Its half-life increases from <3 min to 15 min for 2000-PEG-lactoferrin and 60 min for 4000-PEG-lactoferrin (Table 2). In contrast to carbohydrate receptor clearance, α_2-macroglobulin-protease complexes are cleared through RES receptors for specific sequences of aminoacids on the macroglobulin, which are exposed during complexing with the protease.[12,13] PEG-modification of α_2-macroglobulin-trypsin complexes[12] and PEG-streptokinase complexed to plasmin and α_2-macroglobulin[13] showed significantly longer plasma half-lives than unmodified complexes (although in the latter case some reduced ability to complex to α_2-macroglobulin probably also contributes[13]). In the case of α_2-macroglobulin-streptokinase, it has been shown by competition studies that when the PEG-modified complex is ultimately cleared it is through the same mechanism as that for the unmodified complex.[13] Tissue plasminogen activator (t-PA) is cleared by a nonsaturable hepatic uptake system[17] and, although attachment of a few molecules of PEG to t-PA interfered with its uptake by the liver, a substantial proportion was still cleared by this mechanism.[18]

c. Reduced Proteolysis

An additional advantage of PEG-modification is increased resistance to proteolytic degradation (Tables 3 and 4). The proteases most widely studied include trypsin, chymotrypsin, and *Streptomyces griseus* protease. This resistance to proteolysis presumably reflects steric hindrance of the PEG strands shrouding the protein and preventing proteolytic attack. In the case of trypsin, which cleaves at the carboxy side of lysine and arginine, the presence of PEG covalently attached to lysine represents a direct barrier to cleavage. The extent of modification may relate to the protective effect (cf. the absence of significant protection of streptokinase at 23% modification in contrast with the varying degrees of protection for more highly modified proteins in Tables 3 and 4). However, no formal comparison has been made for a range of proteins to confirm this.

Other factors can contribute to this protective effect. For example, exposure of phenylalanine ammonia-lyase to its competitive inhibitor, cinnamic acid, en-

TABLE 3
Effect of PEG-Modification on Proteolysis by Trypsin

Protein	Lysines modified (%)	Proteolytic digestion (min)	Remaining activity (%)		Ref.
			Native protein	PEG-protein	
Catalase	43	40	0	95	25
Asparaginase	79	10	12	80	192
Streptokinase	23	10	50	50	208
Streptokinase	23	20	18	18	208
β-glucuronidase	60	20	16	83	27
Phenylalanine ammonia-lyase	40	10	17	34	19
•Phenylalanine ammonia-lyase	40	10	17	81	19

• Phenylalanine ammonia-lyase associated with cinnamic acid, a competitive inhibitor of the enzyme.

TABLE 4
Effect of PEG-Modification on Proteolysis by Other Proteases

Protein	Lysines modified (%)	Proteolytic digestion (min)	Remaining activity (%)		Ref.
			Native protein	PEG-protein	
Protease: Chymotrypsin					
Catalase	43	60	30	98	25
β-Glucuronidase	60	20	11	59	27
Protease: Streptomyces griseus					
Catalase	43	60	10	80	25
Phenylalanine ammonia-lyase	40	2	43	68	19
Phenylalanine ammonia-lyase•	40	2	43	79	19

• Phenylalanine ammonia-lyase associated with cinnamic acid, a competitive inhibitor of the enzyme.

hanced the protective effect of PEG-modification although not influencing the proteolytic degradation of the unmodified enzyme.[19] This is somewhat puzzling since it suggests that the cinnamic acid does not merely impede a tryptic cleavage site in the active site region, but must exert some more subtle effect, such as a conformational change, resulting in resistance to digestion at one of the more vulnerable sites in the PEG-modified protein. This effect may be of benefit for *in vitro* as well as *in vivo* applications.

d. Reduced Immunoclearance

Reduced antigenicity and reduced immunogenicity (see below) contribute to lengthened half-lives of proteins after multiple exposures because the half-life of the unmodified protein is often very short and that of the PEG-proteins does not decrease (Figure 1).

2. Reduced Immunogenicity and Antigenicity

Immunological responses limit the clinical use of many proteins. PEG-modification frequently reduces both the antigenicity and immunogenicity of proteins (Tables 5 and 6). Even recombinant human proteins are immunogenic, i.e., they have the ability to invoke antibody formation.[20,21] Such antibodies can neutralize biological activity (if they bind to key epitopes), cause life-threatening hypersensitivity reactions, or increase clearance by the RES.

Reduced antigenicity (i.e., the reduced ability to react with antibodies raised against the unmodified protein and in some cases even the PEG-modified protein) presumably reflects masking of antigenic determinants of the native protein by PEG chains. This often represents a major improvement in clinical settings (cf. the reduction in life-threatening anaphylaxis with PEG-asparaginase[22] and PEG-ADA[23]). One unusual exception to reduced antigenicity is PEG-SOD, which reacted with antibodies to SOD to the same extent as the unmodified enzyme[24] (possibly in this case the antigenic determinant is far apart from a lysine residue).

Markedly reduced immunogenicity is frequently observed for PEG-modified proteins (Table 6). With individual proteins, such as catalase and uricase, the protein is nonimmunogenic only above a certain degree of PEG-modification (58% modification in uricase and 43% in catalase).[25,26] Thus, the mechanism at work may simply be the shielding of antigenic determinants by a material that is relatively immunologically inert, but it may also in part result from avoidance of reticuloendothelial cells.[27] Proteins that dissociate into subunits (and hence reveal new determinants after infusion) may account for the few failures observed with PEG.[19] The route of administration may influence the apparent immunogenicity of the native and/or PEG-modified forms (cf. PEG-1900-catalase, which was nonimmunogenic via the intravenous route and modestly immunogenic via the intramuscular route[25]). When, as rarely occurs, a PEG-protein elicits antibody

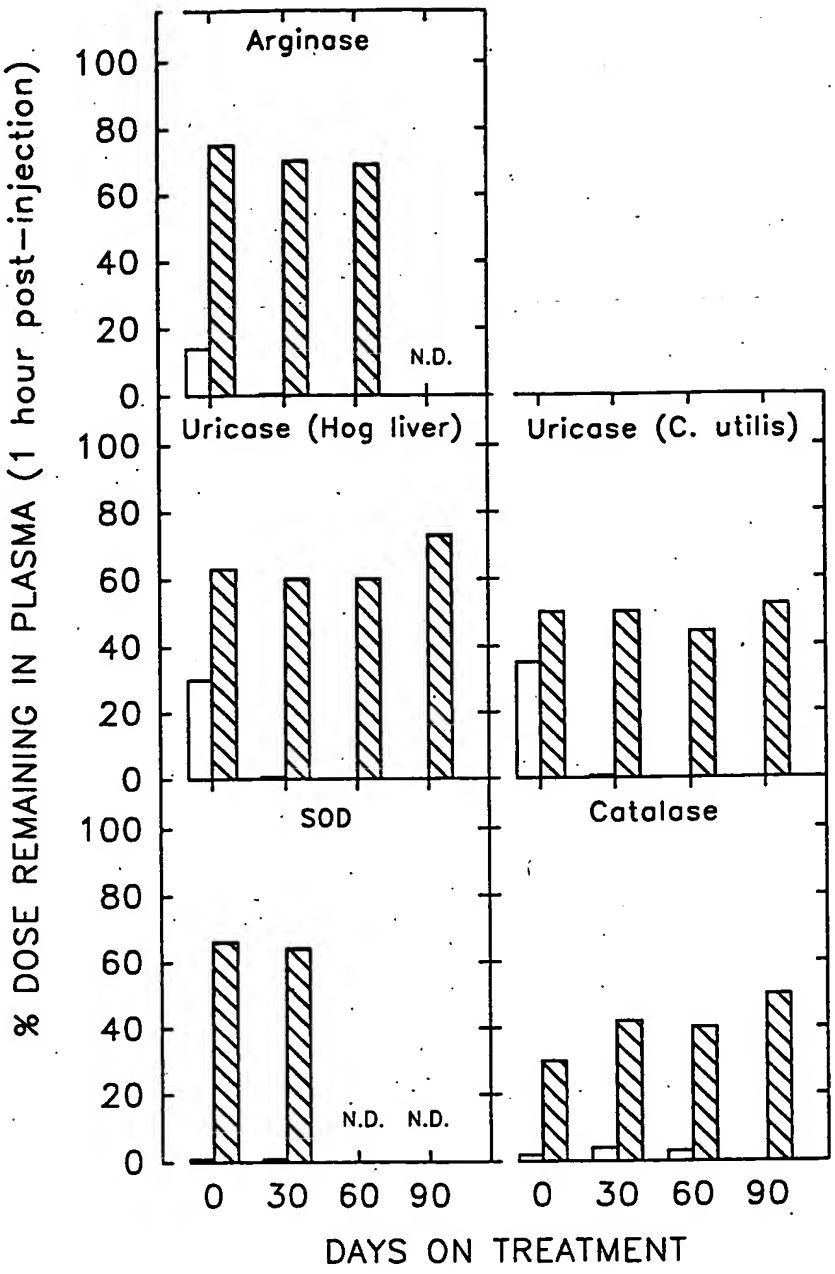

FIGURE 1. Fate of injected unmodified and PEG-modified proteins after single and repeated injections. Mice were subjected to thrice-weekly injections at 2-, 2-, and 3-day intervals. The number of injections received corresponds to the following days of treatment: day 30 = 13th injection, day 60 = 26th injection, and day 90 = 39th injection. The filled and open bars represent modified and unmodified proteins, respectively. Data are taken from: superoxide dismutase,[210] uricase from hog liver,[26] uricase from *C. utilis*,[26] arginase,[59] and catalase.[25]

TABLE 5
Reduced Antigenicity of PEG-Modified Proteins

PEG-protein	Antigenicity	Test system	Ref.
Asparaginase	Abrogated	Precipitin	60, 192
Adenosine deaminase	Abrogated	Immunodiffusion	207
Uricase	Abrogated	Immunodiffusion	26
Uricase	Abrogated	Precipitin	209, 180
Superoxide dismutase	Reduced	Immunodiffusion	50
Catalase	Abrogated	Immunodiffusion	25
Catalase	Reduced	ELISA	24
Arginase	Abrogated	Immunodiffusion	59
Streptokinase	Reduced	R.I.A.*/neutralization	13, 101
Streptokinase	Abrogated	Precipitin	208
β-Glucuronidase	Reduced	Immunodiffusion/ R.I.A.	27
Trypsin	Abrogated	Immunodiffusion	46
Phenylalanine ammonia-lyase	Reduced	Precipitin/ Immunodiffusion	19
Bovine albumin	Abrogated	Immunodiffusion	35

* Radioimmunoassay.

TABLE 6
Reduced Immunogenicity of PEG-Modified Proteins

PEG-protein	Immunogenicity	Test system	Ref.
Asparaginase	Reduced	Mice	72, 60
L-Glutaminase-L-asparginase	Abrogated	Mice	73
Adenosine deaminase	Abrogated	Mice	207
Uricase	Abrogated	Mice	26
Uricase	Reduced	Rabbits	28
Catalase	Reduced	Rabbits	25
Catalase	Reduced	Mice	24
Superoxide dismutase	Reduced	Mice	24
Arginase	Abrogated	Mice	59
β-Glucuronidase	Reduced	Rabbits	27
Phenylalanine ammonia-lyase	Reduced	Rabbits	19
Bovine albumin	Abrogated	Rabbits	35

formation, the antibodies produced seem to be weaker than those produced against the native protein.[19,25]

There is some evidence of cross reactivity between antibodies to PEG-proteins e.g., between anti-PEG-uricase and anti-PEG-SOD antibodies.[28] These antibodies, however, appear not to recognize either PEG itself or the protein; the region of the coupling moiety is the epitope recognized (see coupling methods below). Animals sensitized with antiserum to PEG-uricase prepared with the cyanuric chloride method showed a reduced passive anaphylaxis reaction when challenged with PEG-uricase prepared using the succinimidyl succinate method, which has a different coupling moiety.[28]

PEG on its own is poorly immunogenic and there was production of anti-PEG antibodies in rabbits only after injection of PEG-ovoalbumin in Freund's complete adjuvant.[29] Naturally occurring anti-PEG antibodies have been reported in 0.2% of the normal population and although, following hyposensitization with PEG-honey bee venom and PEG-ragweed extract, antibodies were found in 50% of the patients after the first treatment, there was only a weak IgM response and the patients affected declined to 28.5% after 2 years of treatment. This immunological response is deemed to be of no clinical significance.[30]

3. Increased Solubility

An increased solubility is anticipated for any protein modified with PEG because PEG is highly water-soluble by virtue of hydrogen-bonding of two to three water molecules per ethylene oxide unit.[31,32] Increased solubility has been documented for several proteins.[26,33–35] This is of benefit for both the formulation and administration of proteins with limited solubility at physiological pH, such as some monoclonal antibodies and IL-2 (which precipitates at pH 7 and require either pH 9 or 10 μg SDS per 1 mg of protein to render it soluble[33]). PEG-IL-2 is readily soluble at pH 7, even at 20 mg/ml. Insoluble proteins tend to aggregate when administered and this may be the basis of phlebitis observed at infusion sites[36] and also of rapid clearance.

B. Other Effects of PEG Modification

1. Alteration in Bioactivity

a. Quantitative Changes

The vast majority of PEG-modified proteins show some decrease in bioactivity (Tables 7 and 8). The mechanisms for reduced activity have not been fully addressed and this issue probably has been complicated by the quantitatively different effects produced by different coupling methods. Harris et al.[37] compared PEG-alkaline-phosphatase and found that PEG-cyanuric chloride was most dam-

TABLE 7
Altered Biological Activity of Proteins Modified by PEG-Cyanuric Chloride (PEG-5000)

Protein	Native activity (%)	Substituted amino groups (%)	Method	Ref.
Arginase	65	53	PEG_1-triazine ring	59
Asparaginase	40	18	PEG_1-triazine ring	192
Asparaginase	30	80	PEG_1-triazine ring	192
Asparaginase	8	56	PEG_2-triazine ring	60
Phenylalanine ammonia-lyase	40	28	PEG_1-triazine ring	19
Phenylalanine ammonia-lyase	7	60	PEG_1-triazine ring	19
Uricase	15	43	PEG_1-triazine ring	209
Uricase	45	37	PEG_2-triazine ring	180
Superoxide dismutase	51	95	PEG_1-triazine ring	210
Trypsin	95[a]	24	PEG_1-triazine ring	46
Trypsin	150[a]	59	PEG_1-triazine ring	46

[a] Esterolytic activity.

TABLE 8
Altered Biological Activity of Proteins Modified by Other Activated PEGs

Protein	Native activity (%)	Substituted amino groups (%)	PEG mw	Method	Ref.
Interleukin-2	97	<40	NS[b]	Succinimidyl active ester	15
Interleukin-2	36–71	≥40	NS[b]	Succinimidyl active ester	15
Interleukin-2	140	10–20	5000	Succinimidyl active ester	33
Interleukin-2	47	≥30	5000	Succinimidyl active ester	33
Streptokinase	>100[a]	NS[b]	2000	Carbonyldiimidazole	13
Superoxide dismutase	>95	95	4000	Carbonyldiimidazole	12
Superoxide dismutase	>95	90	5000	Carbonyldiimidazole	12
Superoxide dismutase	80	40	1900	Phenylchloroformate	50
Superoxide dismutase	80	80	1900	Phenylchloroformate	50

[a] Up to about a twofold increase or decrease in activity was observed depending on assay conditions.
[b] Not specified.

aging to the protein, and then, decreasing in order, the carbonyldiimidazole, tresyl chloride, and succinimidyl active ester methods (with 50%, 79%, 84%, and 94% activity at 75% modification, respectively). It seems likely that the best method will vary from protein to protein.

Gross conformational changes do not occur, as shown, for example, by the identity of circular dichroism and ultraviolet spectra of the modified and unmodified proteins.[38,39] Spectroscopic studies on PEG-SOD (^{1}H NMR spectra on the cobalt substituted enzyme) and azide titration have shown that the decrease in enzymatic activity upon surface modification with PEG is not caused by a perturbation of the active site geometry, but to a decrease in the channeling of the superoxide anion towards the enzyme active site.[40] Interference with substrate approach to the active site has also being suggested for deactivation of PEG-alkaline phosphatase.[41] In addition, when increasing the amount of bound PEG there is a decrease in enzyme activity[41] but for the same absolute mass of PEG, PEG-1900 is less deactivating than PEG-5000. Since the former is bound to more lysine residues on the protein it seems that the area of influence of individual PEG chains (determined by flexibility, mobility, and length of the chain) is directly related to the degree of inactivation rather than the modification of a particular residue. This argument is also supported by the observation that attachment of both ends of a PEG molecule to the protein (thus reducing the mobility of the chain) gives less deactivation.[41] In addition, a more compact polymer, dextran (having less mobility), when coupled to uricase produces less deactivation than PEG attached to the same number of lysine residues.[42]

The initial form of the hemoglobin molecule plays a crucial role in the bioactivity of the resulting conjugate (and this could be general for other proteins). Modification of amino groups involved in the formation of salt bridges or in the interaction with 2,3-diphosphoglycerate increases the affinity of the PEG-hemoglobin for oxygen, and this cannot be unloaded from the hemoglobin in the tissues.[43–45] Protection of these amino groups (by using a low pH, circa 6, to protonate the amino groups involved in salt bridges[43] or pyridoxalated hemoglobin to protect the phosphate binding site[44,45]) allows the production of PEG-hemoglobin conjugates with better oxygen-binding properties.

Rare examples show increased activity but most of these have been proteases. Hence, reduced autoproteolysis could account for this phenomenon.[13,46] The rare instances of increased bioactivity for other molecules (such as our demonstration of an increased neutrophil priming activity of PEG-GM-CSF[47]) are intriguing and warrant further investigation. Simple explanations such as failure to internalize and, hence, clear the ligand do not appear to provide an explanation.[48,49]

b. Dissociation of Individual Bioactivities

Somewhat surprisingly, this phenomenon has not only been observed where individual activities of a molecule are spatially separated but also for molecules thought to exert all their pleiotropic activities via a single receptor binding domain.

Examples of the former include PEG-antibodies, where complement fixing activity is lost and antigen-binding is retained after PEG modification of IgG.[38] PEG-ribonuclease has an altered activity for high but not low molecular weight substrates,[50,51] an effect that is simply explained on a steric hindrance basis. Thrombin, a proteolytic enzyme involved in the formation of fibrin and also in the deactivation of the clotting cascade (by switching on protein C) shows a certain dissociation of these two bioactivities upon PEG-modification. Although both fibrin formation activity and potency for protein C activation are reduced, the former is reduced to a greater extent than the latter and the addition of thrombodulin reverts partially only the potency for protein C activation of the conjugate.[52]

However, our observation[47] that PEG-GM-CSF has increased neutrophil priming activity but not increased growth stimulating activity for progenitors (the latter assessed by two independent parameters: colony formation and tritiated thymidine uptake) suggests that the contention that these activities reside in the same portion of the molecule and/or the same type of ligand-receptor interaction may have to be reevaluated. GM-CSF appears both to have a single receptor binding domain[53,54] and to mediate all its actions in progenitor cells and neutrophils via a high affinity receptor.[55,56] The differential effects observed with PEG-GM-CSF may, however, be determined by subtle differences in the conformation of the binding sites of the receptor on progenitor cells and neutrophils, and their respective interactions with the PEG-modified molecule. There are additional potential explanations for our findings: receptor/ligand processing and/or signal transduction may vary for the two biological activities and these in turn could be differentially influenced by PEG modification of ligand. In addition, since progenitor cells and neutrophils produce different proteases,[57] we cannot exclude the possibility of an influence via differential protection from proteolytic cleavage (which we are currently investigating). Whatever the explanation, these findings do raise the novel issue that PEG-modification may yield cytokines with subtly altered spectra of activity and hence different ranges of clinical usefulness.

c. Qualitative Changes

The observation of altered kinetics, reduced temperature optima, broadened pH optima, and changed substrate specificity of enzymes[19,26,46,58] offers the prospect of obtaining beneficial modifications of proteins for some applications. Reduced temperature optima have been observed for catalase,[25] uricase,[26] and phenylalanine ammonia-lyase[19] (however, thermal inactivation of phenylalanine ammonia-lyase above temperature optima was essentially the same for the native and modified enzymes).

Broadening of pH optima to the alkaline range has been observed with phenylalanine ammonia-lyase,[19] β-glucosidase,[19] α-galactosidase,[19] β-glucuronidase,[27] and uricase.[26] It is suggested[19] that the tertiary structure of the protein at higher pHs is stabilized by the attachment of PEG. It may also be relevant that

in all the above examples the attachment of the PEG to the protein occurs via a coupling moiety, the cyanuryl group (see methods below). The linkage of the cyanuryl group (using PEG activated with cyanuric chloride) to the ϵ-amines may alter the local environment of the enzyme, resulting in a catalytically favorable ionic or conformational change, or both, at pH values above the pH optimum.

Where changes in K_m and V_{max} are observed, the former tends to increase and the latter decrease. This is the case with phenylalanine ammonia-lyase,[19] PEG-arginase,[59] and PEG-uricase.[26] The increase in the K_m is not dependent on the degree of modification, i.e., the initial attachment of the first PEG molecule accounts for the increased K_m.[19] The V_{max} decreases as the fraction of modified lysyl groups increases.

However, there are a few exceptions to these changes in K_m and pH optima. There is some controversy about PEG-asparaginase. Kamisaki et al.[60] and Wada et al.[22] did not find changes in the K_m for asparagine and glutamine although Cao et al.[61] report a reduced K_m for glutamine upon PEG-modification. Cao et al.[61] performed the coupling reaction in the presence of asparagine in order to protect the catalytic site, which could explain the discrepancy. PEG-tryptophanase[62] has the same K_m value and the same optimal pH value as the native enzyme. The activation of plasminogen by streptokinase, PEG-2000-streptokinase, and PEG-4000-streptokinase gave comparable K_m values.[13] PEG-β-glucuronidase showed no alteration in the calculated K_m value, but a V_{max} only 20% of that of the native enzyme was observed.[27]

2. Thermal/Mechanical Stability

Proteins tend to aggregate when heated and at air/water interfaces. PEG-modification reduces this inherent aggregateness and may thus reduce denaturation on exposure to heating and/or interfaces. This has been demonstrated for PEG-IgG[38] and is somewhat surprising given the increased surface activity of PEG-IgG (which leads to an increased distribution to the interface, a site usually denaturing). Additional mechanisms may operate in conferring thermostability. The half-life of native lipase at 45°C in water increases from 7.0 ± 0.3 h to 79 ± 9 h upon attachment of PEG-5000. In water, the PEG chains are solvated and may reduce the molecular motions that are intrinsic to denaturation.[63] Thermostability of alkaline proteinase from *Bacillus* sp. is also improved by PEG-modification.[64] However, the thermostability of PEG-ribonuclease is similar to that of the native enzyme[51] and that of PEG-SOD is reduced.[39]

C. Clinical Experience with PEG-Proteins

1. General Experience

To date, the experience with PEG-proteins is limited but encouraging (Table 9). Most evidence suggests that the adducts have not only extended plasma half-

TABLE 9
Clinical Trials with PEG-Proteins

PEG-protein	Trial stage	Patient group	Ref.
L-asparaginase	Phase III	Acute lymphoblastic leukemia	71 204 76
Adenosine deaminase	Phase III	Severe Combined Immunodeficiency Syndrome	75 23 81
Superoxide dismutase	Phase III	Reperfusion injury in renal transplantation	68
Uricase	Phase I/II	Hyperuricemia induced by chemotherapy	82 58
Interleukin-2	Phase I/II	Renal cell carcinoma	211
Catalase	L.P.C.D.*	Trauma, burns, and H_2O_2 injuries	68
Streptokinase	L.P.C.D.*	Thrombolytic therapy	13
Hemoglobin	L.P.C.D.*	Blood substitute and perfusate in organ transplantation	44 99

* Late preclinical development.

lives but improved bioavailability accompanied by improved efficacy. This is particularly encouraging in view of the somewhat reduced specific activity often encountered for PEG-proteins. Since less side effects are observed at lower dosage,[65–67] PEG-modification, by increasing bioavailability and thus allowing dose reduction, may alleviate some toxicity (of course, this will not occur if the desired and undesired effects are intrinsically linked). Reduction in toxicity has been observed with several PEG-modified enzymes.[68] One exception to increased bioavailability is recombinant tissue plasminogen activator, which although when modified has an increased plasma half-life, the protein remaining in the plasma is inactive (probably due to the binding of protease inhibitors).[18] Reduced immunogenicity and antigenicity of PEG-proteins are already producing clinical benefits by decreasing the incidence of life-threatening hypersensitivity reactions.[68] An additional advantage in exploiting the improved pharmacokinetics is the use of bolus injections instead of continuous intravenous infusion.[69]

2. Specific Examples

a. PEG-Asparaginase

PEG-modification improves the therapeutic usefulness of L-asparaginase by increasing its circulation time. In rabbits there is an increase in plasma half-life from 20 h for the native enzyme to 144 h for the PEG-modified L-asparaginase.[70] In four patients receiving chemotherapy for refractory cancers, a single dose of PEG-L-asparaginase with 70% of modification and 52% of enzymic activity had a mean plasma half-life of 22 d compared with a plasma half-life of 7 to 28 h for the native L-asparaginase.[71] The altered pharmacokinetics also include distribution in a volume larger than the plasma compartment, thus providing clinically useful enzyme levels in pleural and peritoneal spaces.[71]

Reduction of the plasma levels of L-asparagine was observed in rabbits after administration of either L-asparaginase or PEG-L-asparaginase. Although levels of L-asparagine returned to normal 4 d after L-asparaginase administration, 27 d elapsed before L-asparagine was detected in rabbits given PEG-L-asparaginase.[70] In four patients receiving chemotherapy for refractory cancers, PEG-L-asparaginase was effective in reducing the plasma asparagine to undetectable levels for as long as the enzyme was detectable in plasma, but no dose-relationship was observed.[71]

PEG-asparaginase shows increased efficacy in several tumor models. One unit of PEG-asparaginase was about as effective as five units of asparaginase against the L5178Y tumor in mice.[72] The survival of BDF_1 mice inoculated with L5178Y lymphosarcoma treated on alternate days for 11 d increases from 11 to 15 d for L-glutaminase-asparaginase to 18 to 22 d with PEG-L-glutaminase-asparaginase (control animals survived 9 to 11 d).[73] Doubling the dosage of unmodified L-glutaminase-L-asparaginase produced a survival time of 18 to 23 d, which is comparable to the survival time of the animals receiving half the dose of PEG-L-glutaminase-L-asparaginase.[73] In C3H/HeJ mice bearing asparaginase-sensitive Gardner lymphoma (6C3HED), PEG-asparaginase was again superior to the native enzyme at all dose levels.[72,74] In this tumor model, i.m. and i.p. routes were assayed and PEG-asparaginase was more effective i.p. than i.m. However, higher i.m. doses were also effective in inducing cures.[72]

PEG-L-asparaginase is currently in phase III trials. In combination chemotherapy, all five patients with second relapse of acute lymphoblastic leukemia (ALL) achieved a third remission after receiving PEG-asparaginase.[75] The remission lasted from 3 to 9 months, median 4 months.[75] PEG-asparaginase was also effective in three children with multiple relapses of ALL allergic to native asparaginase.[76] A recent case report indicates that the optimal clinical dose of L-asparaginase in ALL (6000 to 12,000 IU/m^2 daily or three times a week) could be reduced to 100 to 200 IU once or twice a week using PEG-asparaginase.[77] In two patients with large-cell lymphoma a partial response to treatment was observed.[71] PEG-asparaginase is also more effective than the native enzyme in protecting cells from methotrexate toxicity.[22]

PEG-asparaginase does not always have an improved efficacy. The modified enzyme had the same antitumor activity as the native asparaginase in dogs with non-Hodgkin's lymphoma,[78] and in a recent phase II trial PEG-asparaginase displayed only modest activity in a heterogeneous group of patients with progressive non-Hodgkin's lymphoma.[79] Since both examples are in non-Hodgkin's lymphoma, the lack of improved efficacy may be related to this particular setting (although in a recent report some success was observed in a patient diagnosed as non-Hodgkin's lymphoma stage III[77]).

The use of L-asparaginase is usually complicated by immunological reactions but these are reduced by PEG-modification. While the antitumor activity of L-asparaginase is prevented in an animal model by preimmunization with the native enzyme, this did not affect the therapeutic efficacy of the PEG-asparaginase against lymphoma.[74] Life-threatening hypersensitivity reactions are significantly reduced by PEG-modification of the L-asparaginase.[68] Only 3 out of 22 patients treated with PEG-L-asparaginase had hypersensitivity reactions.[68] In addition, children known to be allergic to asparaginase successfully continued treatment with PEG-asparaginase and no anaphylactic reactions were observed.[75,76] PEG-asparaginase was nonimmunogenic, nonantigenic, and nonallergenic when given to four patients with lymphoma or small cell carcinoma of the lung, which is refractory to conventional therapy.[71]

In general, the toxicity record of PEG-asparaginase is superior to that of L-asparaginase. PEG-asparaginase induced only low-grade toxicosis[80] (except *Achromobacter* asparaginase, which proved to by unacceptably toxic in an early phase I trial[71]). Toxicity in five patients with second relapse of acute lymphoblastic leukemia was transient and mild.[75] In dogs with non-Hodgkin's lymphoma, PEG-asparaginase lacked all the side effects of the native enzyme.[78] Transient elevations of hepatic enzymes, but no overt hepatic toxicity, have been observed. Nausea, vomiting, fever, and malaise have occurred.[68] PEG-L-asparaginase (*E. coli*) (70% of modification) showed minimal toxicity when given to a group of four patients with lymphoma or small cell carcinoma of the lung. There were no episodes of hyperglycemia or hypocalcemia and no neurologic toxicity.[71]

b. PEG-Adenosine-Deaminase (ADA)

PEG-ADA is in phase III trials and has FDA approval. PEG-ADA is used in ADA-deficient Severe Combined Immunodeficiency Syndrome.[68] ADA itself is too rapidly cleared to be used therapeutically and the current practice involves red blood cells transfusions. The modified enzyme was rapidly absorbed after intramuscular injection in two children and provided and maintained levels of plasma ADA two to three times the normal level of erythrocyte ADA with a weekly dose of 15 U per kilogram of body weight.[23] The principal biochemical consequences of ADA deficiency (decrease in adenosine nucleotides, increase in deoxyadenosine nucleotides; low or absent activity of s-adenosyl-homocysteine) were almost completely reversed.[23] Neither toxic effects nor hypersensitivity reactions nor infections were observed.[23] PEG-ADA also has been successful in

the treatment of a 5-year-old girl who was asymptomatic during the first years of life.[81] Prolonged treatments with PEG-ADA seem not be a problem inasmuch as the only adverse effects reported in a patient who has now received PEG-ADA therapy for 3 years have been a headache and transient pain at the injection site.[68]

c. PEG-Uricase

PEG-uricase is a uricolytic agent in phase I trials for treatment of hyperuricemia associated with chemotherapy.[68] After the injection of PEG-uricase in a patient with non-Hodgkin's lymphoma, uricase activity appeared rapidly in plasma, peaking within 24 h and persisting for approximately 5 d. An inverse relation between plasma uricase activity and plasma urate concentration was reported.[82]

PEG-uricase is well tolerated and mild pain at the injection site was the only adverse effect reported.[82] PEG-uricase given to five men with advanced hematological malignancies elicited no toxic effects and there was a rapid disappearance of serum uric acid for over 32 h.[58] Uricase was ineffective in lowering plasma urate levels after injection with four-weekly doses of enzyme into Leghorn cockerels but PEG-uricase, after multiple doses, was as effective with the first injection.[83]

d. PEG-Interleukin-2 (IL-2)

PEG-rIL-2 was approximately 60 times more potent than rIL-2 as indicated by 0.4 μg of PEG-rIL-2 having had effects comparable to 25 μg of unmodified material in a murine tumor model.[33] Both rIL-2 and PEG-rIL-2 inhibited the tumor growth in a dose-dependent manner. All doses were well tolerated. At equitoxic dose levels, the antitumor activity of PEG-IL-2 was far superior to that of rhIL-2 in three transplantable syngeneic murine tumor models, i.e., Meth A fibrosarcoma, B16 melanoma, and Pan-02 pancreatic carcinoma.[84] Efficacy of PEG-IL-2 was highly dependent on the dose and also on the schedule of administration, being best with the Q7Dx2 (twice every 7 d) schedule than several alternative schedules (Q4Dx3, Q3Dx4, Q2Dx5) where either the efficacy was reduced or the toxicity of the treatments was increased.[84] PEG-modification also reduced the immunogenicity of IL-2.[85]

e. PEG-Superoxide-Dismutase (SOD) and PEG-Catalase

PEG-SOD and PEG-catalase potentially have an important role for both diagnosis and treatment of pathologies involving free radicals.

Partial protection against streptozotocin-induced hyperglycemia by PEG-SOD suggests that superoxide radicals may be involved in such a diabetogenic action.[86] PEG-SOD has been assessed for improving the survival of skin flaps. Control flaps were found to have a survival rate of 40%, native SOD (20,000 U/kg IV) improved this to 52%, but PEG-SOD achieved 80% survival.[87] PEG-SOD pre-

vents lipid peroxidation in burn patients.[88] In myocardial infarction on animal models, native SOD was without effect whereas PEG-SOD significantly reduced the percent-infarcted area and area of induced ischemia.[89] Both PEG-SOD and PEG-catalase reduce infarct volume in cerebral ischemia.[90] PEG-SOD was more effective in kidney preservation (preserved by hypothermic perfusion) as shown by a significant improvement in creatinine clearance (330 ± 58 ml/h) compared with the unmodified enzyme (261 ± 82 ml/h) when used as constituents of the perfusate to act as free radical scavengers.[91]

Combined treatment with PEG-SOD and PEG-catalase prevents the pulmonary oxygen toxicity due to hyperoxia in rats[92] and high oxygen concentrations and high mean airway pressures during mechanical ventilation of premature newborn lambs.[93,94] PEG-SOD has also been used in unilateral lung injury due to reexpansion (reoxygenation).[95]

Despite these encouraging results, PEG-catalase failed as an antioxidant in acute pancreatitis due to the inability of the modified enzyme to reach extravascular sites.[96] This fact could be related to an enhanced association of PEG-catalase with endothelial cells,[97] which may impose a limitation to the potential uses of the modified enzyme.

Reduced side effects have been reported for PEG-SOD. Interarticular injection of unmodified SOD into arthritic joints is sometimes followed by swelling and local irritation, whereas PEG-SOD does not elicit these reactions.[68] Preclinical animal studies of PEG-catalase only showed adverse effects at 2000 to 4000 times the human recommended dose in mice and no toxicity was observed at 20 times the human recommended dose in rats.[98] Histopathological examination of 25 tissues (hematoxylin and eosin stains) in three mice after 90 d of injection with PEG-catalase showed that all tissues were within normal limits.[25]

f. Other PEG-Modified Conjugates (Preclinical Studies)

PEG-hemoglobin is showing promise as a blood substitute because the conjugate retains two essential functions: maintenance of blood volume and tissue oxygenation.[10,43,44] PEG-hemoglobin also has an added value as a perfusate for organ preservation.[99] In an animal model, liver preservation for 24 h with PEG-pyridoxylated-human-hemoglobin recovered complete function after transplantation, leading to longer survival.[99]

PEG-bilirubin oxidase, in a bolus injection, rapidly and substantially decreased plasma bilirubin levels in bile-duct-ligated jaundiced rats and the effect persisted for longer than 3 h.[100] This rapid and persistent decrease in plasma bilirubin levels makes PEG-bilirubin oxidase a valuable tool for both the study and treatment of bilirubin toxicity in jaundiced animals.

PEG-streptokinase and PEG-tissue plasminogen activator show promise as thrombolytic drugs.[18,101,102] All the generic benefits of PEG-modification have been found in PEG-streptokinase,[13,102] PEG-acyl-streptokinase,[101] and PEG-tissue plasminogen activator conjugates.[18] PEG-urokinase (a human urinary plasmino-

gen activator) in an *ex vivo* study using beagles was found to maintain urokinase activity in the blood after intravenous injection. Also, fibrinolysis was activated more by PEG-urokinase than by native urokinase.[103] PEG-batroxobin (a venom produced by *Bathrops atrox* that potentiates the fibrinolytic system in the blood) was administered to dogs and efficiently decreased the blood fibrinogen level, thus increasing the fibrinogen degradation products in the blood.[104] PEG-modification of serpins, α-1-antichymotrypsin and α-1-antitrypsin (also known as α-1-antiproteinase), improves their therapeutic potential.[105] Plasma-derived α-1-antiproteinase, when coupled to PEG, is cleared from the plasma much more slowly, and it can still be metabolized in a manner similar to the unmodified protein and forms complexes with proteinases (elastase) which are catabolized by the same hepatocyte receptor that binds α-1-antiproteinase-proteinase complexes. Thus, the PEG-modified α-1-antiproteinase is of value for replacement therapy in patients with emphysema due to enzyme deficiency.[106]

PEG-F $(ab')_2$ fragment of monoclonal antibodies with affinity for tumor cells appears to be a promising carrier for use in targeting cancer chemotherapy.[107,108]

3. PEG-Protein Conjugates as Tolerogens

PEG-modified proteins can also be used to generate tolerance.[109–113] For this application, a narrow window has been observed for the number of PEG molecules which, when attached, will induce tolerance to the protein (PEG-protein conjugates modified to either a higher or a lesser extent are ineffective).[113] This observation might be the basis for the few failures observed in early studies (e.g., uricase[26] and arginase[59]). Induction of tolerance has been reported in both unsensitized and sensitized mice.[114]

The primary and ongoing IgE responses to the corresponding native allergens are abrogated whereas the ongoing IgG antibody production is less affected (the latter were unchanged or enhanced with PEG-honey-bee venom[115]). The suppression of IgE antibodies in allergic states appears to be due to less effective presentation of antigenic determinants to helper T cells,[116] induction of suppressor T cells,[117] and activation of specific suppressor T cells with release of suppressor lymphokines.[118] A decrease in mast cell sensitivity also has been observed with PEG-ovoalbumin and PEG-ragweed pollen extract.[119] Tolerance induced in mice to human monoclonal IgG (HIgG) by injection of PEG-HIgG was transferred to normal mice by T cells and T cell extracts (carry-over of the tolerogen with the transferred cells has been totally excluded)[117,120,121] and by suppressor lymphokines released from these cells.[118]

D. Toxicology of PEG

PEG is approved by the FDA for parenteral use, topical application, and as a constituent of suppositories, nasal sprays, foods, and cosmetics.[68] PEG is of low toxicity when administered orally or parenterally[122] and only large quantities

invoke adverse reactions. A diet containing 16%, w/w, PEG-6000 is harmless to rats.[123] PEG grafted to cellulose hemodialysis membranes suppresses blood and membrane interaction and thus improves biocompatibility and reduces thrombogenicity in clinical hemodialysis.[124]

Toxicities of PEG have been observed in two settings using low-molecular-weight PEG: after absorption of topically applied PEG and when PEG was used to test intestinal absorption. Significant amounts of topically applied low-molecular-weight PEG are absorbed and this has been implicated in the deaths of burn patients treated with PEG-based antimicrobial creams.[125] These patients developed renal failure associated with metabolic acidosis, i.e., elevated anion and osmolality gaps, with a raised total calcium but normal or decreased ionized calcium.

Subsequent studies in rabbits confirmed the suggestion that it was the PEG-content of the cream that was responsible for the metabolic abnormalities, with the possible exception of the osmolality gap.[126] Mass spectrometric data confirm the presence of glycol metabolites (hydroxyglycollic acids and diglycollic acid homologues) in serum and urine of patients treated topically with PEG.[127] These carboxylic acids cause the metabolic acidosis. Both the mono- and diacids formed are potent chelators of calcium,[128] causing calcium mobilization to maintain the level of ionized calcium. The metabolic products of PEG, acids and aldehydes, are toxic to renal epithelial cells[129] thus exacerbating the metabolic abnormalities but reducing clearance of PEG and its metabolites. While these studies yielded important information about the metabolism of PEG, such toxicities are unlikely to be relevant to the administration of PEG-modified cytokines and enzymes because only chronic administration of large quantities of a PEG-modified protein are likely to induce significant toxic effects. Experience with intravenously administered PEG-cytokines and PEG-enzymes (see above) bears this out.

III. *IN VITRO* APPLICATIONS OF PEG-PROTEINS

A. PEG-Proteins in Aqueous Two-Phase Systems

Aqueous two-phase systems formed by either PEG and dextran or PEG and phosphate have been extensively used for the isolation and/or fractionation of biological material, including cells, organelles, proteins, and nucleic acids.[130–132] Differential partition of the target and contaminant materials into the top phase (PEG-rich) and the bottom phase (dextran-rich or phosphate-rich) is the basis for the separation. Proteins covalently modified with PEG partition almost exclusively into the top PEG-rich phase and, thus, PEG-proteins are uniquely tailored to be used as bioaffinity ligands to specifically increase the partitioning of the target material into the top phase.

1. Preparative Affinity Phase Partitioning

Affinity phase partitioning has been used preparatively for the isolation of cells,[133–137] subcellular fractions,[138] and DNA.[139] The proteins used to produce

the PEG-ligands include antibodies,[133,134,137] protein A,[135] streptavidin,[138] transferrin,[140] and enzymes.[139]

a. Immunoaffinity Phase Partitioning

Several strategies have been employed to immunospecifically increase the partitioning of target cells. The first examples of immunoaffinity cell partitioning (IACP) used a single antibody system (primary antibody) in which antibodies to the target antigen are used to prepare the affinity ligand.[133,134,137] Double "layer" systems have also been reported. For example, secondary antibodies[136,141] and protein A[135,142] covalently linked to PEG had been used as general affinity ligands for the extraction of cells selectively coated with a primary antibody. PEG-streptavidin has been used in conjunction with biotinylated antibodies directed against the target antigen.[138]

The increase in partition of the target cells is related to the amount of PEG coating the cell and the composition of the biphasic system. The former, in turn, depends on the concentration of PEG-antibody,[133,134,137] the expression of the antigen,[135,136,142] and the degree of PEG-modification of the antibody.[133,134] These three parameters will determine the number of PEG-antibody molecules bound per cell. There is a window for the optimum number of PEG chains attached per antibody molecule: the larger the number, the lower the affinity of the PEG-antibody conjugate for the antigen and, thus, less occupancy at a given concentration; on the other hand, this negative effect is compensated for with the loss of agglutinating ability by adding more PEG chains, thus reducing clumping of the cells which then settle to the interface.[133,134,137]

The versatility of IACP has been demonstrated empirically using model systems. These include positive selection of human erythrocytes from mixtures with sheep[133] and rabbit[134] erythrocytes. Thirty extractions with the countercurrent distribution (CCD) procedure led to complete separation of the cell mixtures.[133,134] In all the examples, 100% of the cells of interest were recovered after the procedure.[133–136] IACP can discriminate cells on the basis of the surface expression of a receptor, as shown in a model system with two subsets of a virally-transformed mouse lymphocyte line, MBL-2(4.1) and MBL-2(2.6), having 3×10^9 and 1×10^9 antigen binding sites per square centimeter for the affinity ligand rat monoclonal IgG(YEI 48.10).[141] Recently strategies to isolate a cell population present at low abundance (<1%) in good yield have been devised.[137]

b. PEG-Ligand Phase Partitioning

Ligand-receptor interactions have also been exploited to manipulate the partitioning behavior of a selected cell population.[140] An advantage over the immunoaffinity approach is the use of a ligand that occurs naturally, rather than antibodies which have to be produced for each single antigen.

Using PEG-transferrin as the ligand and rat reticulocytes as the target cells, the increase in the partition of the reticulocytes takes place within a narrow range of MPEG-transferrin bound per cell, 10.2 to 11.3 fg/cell. The latter figures correspond to approximately 80,000 and 89,000 molecules of MPEG-transferrin bound per cell, respectively.[140]

c. PEG-Enzyme Phase Partitioning

PEG-modification of proteins covalently attached to DNA has been exploited to devise a purification method for DNA/topoisomerase II complexes with which to examine the cleavage site specificity of the enzyme in cellular differentiation.[139] The principle of this method is to couple PEG to proteins while they are attached to DNA (topoisomerase II forms a covalent DNA complex as it acts[143,144]) and then to use phase partitioning in an aqueous two-phase system of PEG and phosphate to separate free DNA from DNA bound to PEG-modified proteins (which have high affinities for the phosphate-rich and PEG-rich phases, respectively).

Applying this PEG-enzyme phase partitioning method to genomic DNA obtained by SDS/KCl precipitation[145] from HL60 cells induced to differentiate to the granulocyte lineage (by retinoic acid) or to the monocyte/macrophage lineage (by phorbol myristate acetate), it has been demonstrated that specific DNA sequences become protein-linked, probably to topoisomerase II, during induced differentiation.[139] This observation is of great significance because previous methods (including SDS/KCl precipitation) failed to recover more DNA from cells induced to differentiate, compared with the controls.[145]

2. Analytical Affinity Phase Partitioning

Phase partitioning had been exploited in binding assays to separate free from bound ligand (taking advantage of spontaneous asymmetrical partition of both components) in order to calculate apparent binding constants.[146–149] The construction of PEG-proteins by altering the partitioning behavior of the protein has made it possible to extend this methodology to ligand-receptor systems where the separation of free and bound ligand is not very efficient or does not occur.[150–153] The use of PEG-modified proteins in either competitive or direct binding assays, in combination with phase partitioning, thus offers a new possibility when designing assays based on biospecific interactions between ligands and receptors, antigens and antibodies, substrates and enzymes, carbohydrates and lectins, etc.

B. PEG-Enzymes in Organic Solvents

The amphipathic nature of the PEG molecule confers to PEG-proteins solubility in hydrophobic solutions, including organic solvents.[154–156] Interestingly,

PEG-enzymes solubilized in organic solvents retain enzymic activity and this creates a number of potential applications. PEG-enzymes can be used as tools to understand enzymic reactions occurring in a hydrophobic environment which otherwise are unaccessible. In a "low-water" environment, enzymes with hydrolytic activity efficiently catalyze the reverse reactions, i.e., synthesis, and this may be of extreme importance because it would allow stereospecific synthesis of various organic compounds with yields and purities far above those expected by classical synthesis (see below). In some cases, hydrolytic activity is retained in organic solvents.[64]

Quantitative analysis of compounds insoluble in aqueous systems can be performed in organic solvents by using the appropriate PEG-modified enzyme. The recovery of active enzyme be precipitation from the organic solvent (e.g., PEG-lipase is recovered active from benzene by addition of n-hexane or petroleum ether[157]) makes PEG-enzymes very attractive for biotechnological applications.

The basis for the catalysis in organic solvents seems to lie in water molecules being bound to the PEG to provide the appropriate environment for the functional enzyme.[158–160] There seems to be an optimum concentration for water for each reaction.

1. PEG-Enzymes as Catalysts in Organic Solvents

PEG-chymotrypsin catalyzes the aminolysis of esters to form peptide bonds in benzene, and the reaction occurs quantitatively without any ester hydrolysis. This is an advantage over the reaction synthesis in aqueous solution where the competition between aminolysis and hydrolysis reduces the yield of the peptide.[158,161] The distinct specificity of the PEG-chymotrypsin for ester donor and amine acceptor[161] allows the production of the desired dipeptide (e.g., N-benzoyl-tyrosine-phenylalanine-ethyl ester) without any secondary reaction leading to the formation of unwanted dipeptides (phenylalanine-phenylalanine).

PEG-lipase catalyzes not only the reverse reaction of hydrolysis, ester synthesis,[63,159,162] but also reactions of ester exchange and aminolysis in benzene.[159,163] The latter reaction is of special interest since the use of nonproteases for peptide synthesis overcomes the problems inherent to proteases: narrow substrate specificity and undesirable proteolysis of the growing peptide chain.[164] Ester-exchange reactions occur efficiently between an ester and an alcohol, an ester and an acid, and between two esters, both in organic solvents and in straight hydrophobic substrates.[163] One of the applications is in the reformation of fat to oil by introducing unsaturated fatty acid into triglycerides to lower the melting point (this will be useful in the food industry).[165] PEG-lipase efficiently catalyzes the synthesis of polyester in benzene using 10-hydroxydecanoic acid as substrate.[166]

PEG-horseradish-peroxidase[167] and PEG-hemin[168] conserve the peroxidase activity in organic solvents and thus can be used as indicators of reactions producing hydrogen peroxide. PEG-cholesterol oxidase efficiently catalyzed the oxidation of cholesterol in benzene, and the rate of cholesterol-oxidation could

be determined spectrophotometrically by coupling the reaction to oxidation of o-phenylenediamine by PEG-peroxidase.[169] PEG-catalase can be used to decompose hydrogen peroxide in organic solvents.[170]

2. New Properties in Organic Solvents

In general, PEG-proteins show increased solubility in a number of organic solvents such as benzene, toluene, and dimethylsulfoxide.[63,159,163,170] Their catalytical activity is clearly demonstrated by the linear relationship between rate of reaction and amount of enzyme added.[159,163,169] Maintenance of the catalytical activity suggests that PEG-modification does not alter the active conformation of the enzyme. Spectroscopic studies show that the prosthetic group is not liberated from the apoprotein in PEG-horseradish peroxidase,[167] PEG-catalase,[170] and PEG-cholesterol oxidase[169] after solubilization in benzene.

The degree of modification has quantitatively different effects on the solubility and activity of the PEG-enzymes in organic solvents. There is an increase in solubility with increasing degree of modification of PEG-catalase, although the enzyme activity reaches a maximum at some intermediate solubility.[170]

The relative catalytic efficiency of PEG-enzymes in organic solvents can not always be established because for some of them the only activity measurable for the native enzyme is in the reverse reaction in water. The assessment is also complicated by the influence on PEG-enzymes activity by different organic solvents (in general, higher activities in water-immiscible than in water-miscible solvents).[158,159] In a comparison of enzymic activity in water vs. organic solvents, PEG-horseradish peroxidase in benzene had only 21% of the activity of the native enzyme in aqueous solution.[167] By contrast, other examples showed an increase in activity. PEG-hemin is more active in the peroxidase reaction in 1,1,1-trichloroethane than unmodified hemin in aqueous solution,[168] and PEG-catalase in benzene has more activity than catalase in water.[170] PEG-lipase is more active than native lipase in both hydrolytic and synthetic reactions in organic solvents, whereas as hydrolytic enzymes in water, native lipase is more active than PEG-lipase.[63] The enhanced activities could not be attributed to an increased stability. Stability of PEG-lipase and lipase varies in the following order at 45°C:

$$\text{PEG-lipase}_{\text{water}} > \text{lipase}_{\text{benzene}} > \text{PEG-lipase}_{\text{benzene}} > \text{lipase}_{\text{water}}{}^{63}$$

This behavior was not unexpected because solubilizing the enzyme with PEG would introduce flexibility in organic solutions, thus lowering the stability, whereas in water the solvated PEG chains may reduce the molecular motions that are intrinsic to denaturation.[63] PEG-lipase was, however, extremely stable at elevated temperatures (70°C)[163] and efficiently catalyzed ester synthesis at −3°C.[171]

The specificity of PEG-enzymes for substrates in organic solvents is not always comparable to that of the unmodified enzyme in aqueous solutions and this leads to unsuspected new reactions. The acceptor nucleophile specificity of

PEG-chymotrypsin in organic solvents was comparable to that in water whereas its substrate specificities in organic solvents and in water were different because, besides aromatic aminoacids, arginine and lysine esters were found to be effective in forming the acyl-enzyme with subsequent synthesis of a peptide bond.[158] The specificity of PEG-lipase is also altered with respect to native lipase.[63] A potentially important observation is the distinct specificity for PEG-lipases produced with enzymes from different sources, e.g., lipases from *C. rugosa* and *P. fluorescens*.[172,173]

Finally, the kinetics of the reactions in hydrolytic and synthetic modes seems to be different. The rate-limiting step in chymotrypsin-catalyzed hydrolysis of amide bonds is the formation of the acyl-enzyme, whereas in organic solvents deacylation is the rate-determining step in chymotrypsin-catalyzed synthesis of peptide bonds.[158]

IV. PEG COUPLING METHODS

Despite the well-established advantages of PEG-proteins over their unmodified counterparts, this technology has not yet been very widely exploited commercially because until recently most methods were suboptimal: they use conditions which are likely to reduce biological activity, they are not readily controlled with respect to the number of PEG molecules attached, and they often require purification steps which themselves endanger labile proteins. They also leave part of the coupling moiety attached to the protein, where it may serve as an antigen (see above). Newer methods circumvent these problems and should encourage rapid progress.

A. General Principles

In general, all the methods take advantage of the nucleophilic nature of the N atom in the ϵ-amino group of lysine. There are two strategies to produce the coupling. In both, there is activation of the hydroxyl group(s) of the PEG with a group, the coupling moiety, susceptible to nucleophilic attack by the N atom of the amino group. Generally, monomethoxyPEG (MPEG) with only one available hydroxyl group is selected so that coupling can only occur at one end of the PEG molecule. In some methods this nucleophilic attack results in the coupling moiety being incorporated as part of the PEG-protein adduct whereas with others all the activating group is lost, resulting in direct coupling of PEG to the protein (this, of course, depends on the site of the atom susceptible to nucleophilic attack). For an extensive review of methods to activate PEG see Buckman and Morr,[174] Harris et al.,[175] Harris,[176] and Zalipsky and Lee.[177]

B. Specific Methods

The major coupling methods are illustrated in Figures 2 to 10. Most have been available for several years. PEG activated with cyanuric chloride was the first polymer used to couple PEG to proteins.[25,35] Other methods were developed afterwards in order to avoid the adverse coupling conditions (unphysiological pH and salt composition), and possibly toxic coupling moiety and degradation products, of this activated polymer. The alternative methods more frequently used are activation with 1,1′-carbonyldiimidazole,[12] phenylchloroformates,[50] and succinimidyl active ester.[37] Each method has its own inherent problems.

Cyanuric chloride (Figure 2) has been the most widely used agent for activation of PEG. The classical approach couples one triazine ring per PEG molecule (Figure 2a). This activated polymer uses unphysiological conditions for coupling to protein (0.1 M sodium tetraborate pH 9.2).[25,178] It probably destroys more biological activity than the other major methods.[37] Because there are two chlorines in the triazine ring and each is potentially reactive, cross-linked products can be formed. This seems likely to be the case for hemoglobin because the increase in molecular mass, assessed by gel filtration, is about 750,000 Da (i.e., much higher

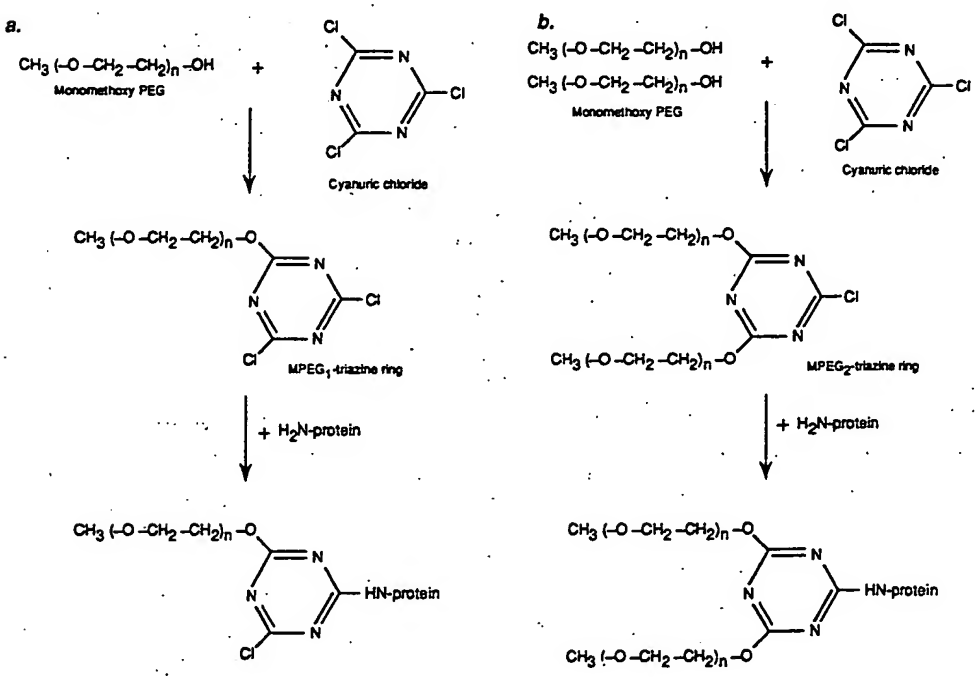

FIGURE 2. Activation of MPEG with cyanuric chloride and its attachment to amino groups in a protein: *a.* $MPEG_1$-triazine ring (adapted from Abuchowski et al.[35]); *b.* $MPEG_2$-triazine ring (adapted from Yoshimoto et al.[72]).

than expected for a single Hb molecule).[43] Similar cross-linking was observed after reaction with 2-methoxy-4,6-dichloro-s-triazine (i.e., a coupling moiety in which PEG is replaced by a methyl group).[43] In contrast, using a succinimidyl active ester (i.e., with no additional site for reaction) the apparent molecular mass of PEG-Hb conjugates is 160,000 to 200,000 Da.[43,179]

To avoid crosslinking when using a cyanuric chloride-activated PEG, Inada et al.[76] prepared 2,4-*bis*(*O*-methoxypolyethylene glycol)-6-chloro-s-triazine, which leaves only one reactive chlorine per triazine ring (Figure 2b). A second advantage is that the PEG_2-triazine ring seems to be more effective in rendering proteins nonimmunogenic without compromising the biological activity as much as the PEG_1-triazine ring.[76,180] Inada et al.[76] demonstrated the following: (1) an increased molecular weight of the PEG_2-triazine ring polymer when compared with PEG_1-triazine ring polymer (this seems to support that the former contains more PEG chains per triazine ring than the latter), and (2) modification of proteins with the heavier PEG_2-triazine polymer. However, the reactivity of this polymer has been questioned.[177] Jackson et al.[178] reported lack of reactivity for the third chlorine (the only chlorine available in PEG_2-triazine ring) based on elemental analysis of the preparations obtained by reaction of PEG_1-triazine ring with ethanolamine or by hydrolysis in water. Unfortunately, elemental analysis of the preparations does not provide information on the number of species present. Therefore, it has not been proved whether the preparations consisted of only a single compound in which only one of the chlorines is reactive (e.g., PEG_1-triazine ring-ethanolamine) or, by contrast, it consisted of a mixture containing PEG_1-triazine ring (25%), PEG_1-triazine ring-ethanolamine (50%), and PEG_1-triazine ring-ethanolamine$_2$ (25%) (this is a statistical mixture if the two chlorines left in PEG_1-triazine ring are equally reactive). The cross-linked products alluded to above are sufficient evidence for the reactivity of the third chlorine.

Other disadvantages of PEGs activated by cyanuric chloride are, however, more difficult to avoid. Cyanuric chloride can couple to SH groups and this may be responsible for enzymatic inactivation.[19,62] Cyanuric chloride is toxic per se and its degradation products may also be toxic.[50]

Succinimidyl active ester methods include a variety of allied methods. Either a carboxylic acid is generated by addition of an anhydride (e.g., succinic anhydride as shown in Figure 3) or the carbinol function ($-CH_2OH$) of the PEG is converted to a carboxylic acid by a three- or four-step procedure or a two-step oxidation (reviewed by Veronese et al.[50]). This is followed by activation with *N*-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide (DCC). PEG-*N*-succinimidyl-succinate has been used to couple PEG to asparaginase,[72] adenosine deaminase,[23] and uricase.[28] PEG-*N*-succinimidyl-glutarate has been used to couple PEG to IL-2.[33] Unlike the cyanuric chloride method, these methods yield activated PEGs that do not inactivate SH-dependent enzymes.[50] However, these coupling methods are limited by the easy hydrolysis of the ester between PEG and the acid (succinic acid, glutaric acid, etc.).[50] To avoid the ester labile site, the carboxylic acid has to be generated without addition of an anhydride but with conversion of the carbinol function of the PEG.[50] A complex version of these

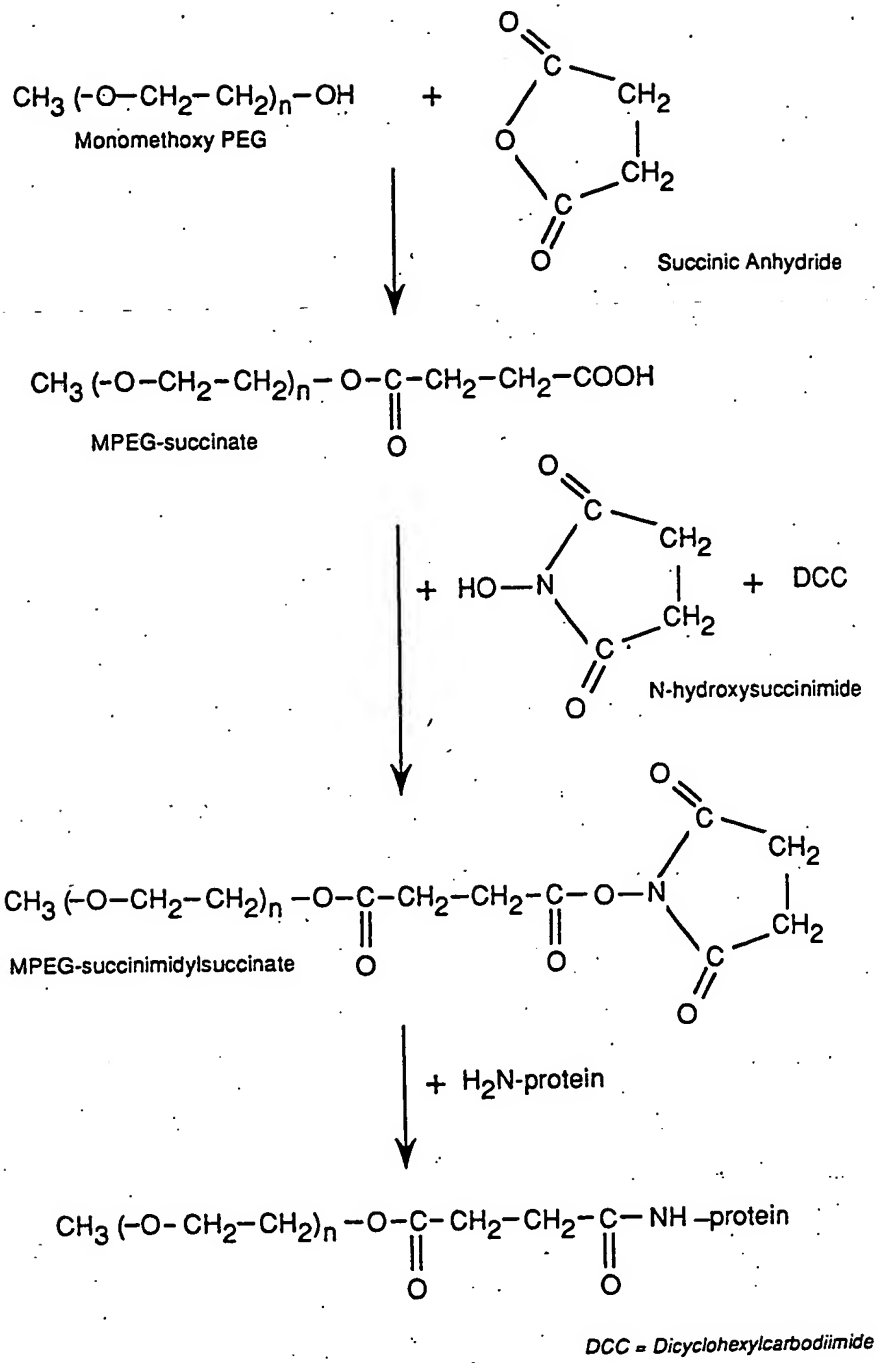

FIGURE 3. An example of succinimidyl active ester methods (succinimidyl succinate) for activation of MPEG and subsequent coupling to a protein (adapted from Abuchowski et al.[72]).

methods binds PEG-succinimidyl succinate to nor-leucine, activates the carboxyl group in nor-leucine with *N*-hydroxysuccinimide, crosslinks the resulting polymer with lysine, and activates the carboxyl group of the lysine with *N*-hydroxysuccinimide to obtain the activated polymer for reaction with the target protein.[181]

The carbonyldiimidazole method[12] uses a long reaction time of up to 72 h and unphysiological conditions (e.g., pH 8.5) to couple the polymer to the protein. 1,1′-Carbonyldiimidazole reacts with the hydroxyl group of PEG to form an imidazole carbamate derivative. The presumed reaction is shown in Figure 4. In direct comparison of bioactivity of PEG-alkaline phosphatase, this method has an intermediate performance between the cyanuric chloride method and succinimidyl active ester methods.[37] However, PEG-tissue plasminogen activator prepared by this method retained more activity than the conjugate prepared with a succimidyl active ester method.[18] Biologically active PEG-streptokinase[13,102] and

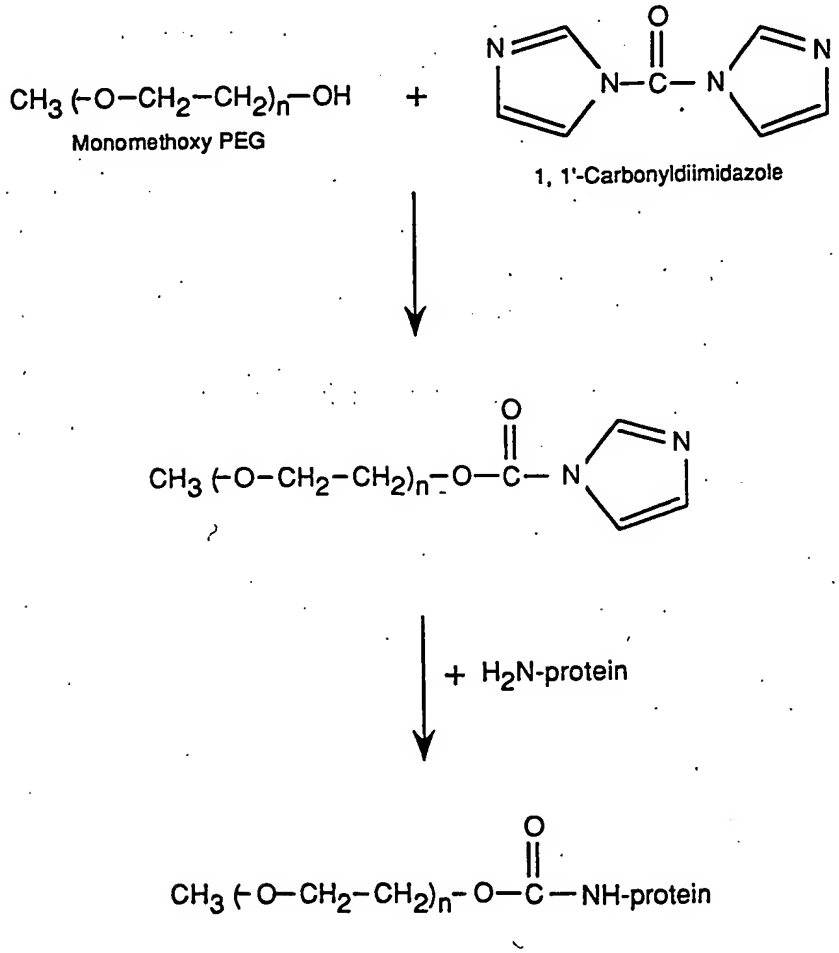

FIGURE 4. Activation of MPEG with 1,1′-carbonyldiimidazole and its attachment to amino groups in a protein (adapted from Rajagopalan et al.[76]).

PEG-SOD[12] have been successfully constructed using the carbonyldiimidazole method.

Phenylchloroformate methods require a very high pH (e.g., 8.5 to 9.2) for the coupling step and do not appear to be widely used.[50] Two examples are shown in Figure 5. In Figure 5a, PEG-2,4,5-trichlorophenylcarbonate is the active species and in Figure 5b it is PEG-p-nitrophenylcarbonate. These intermediates are unstable at alkaline pH, giving appreciable hydrolysis over the time scale of PEG reacting with the protein. Once made, the activated PEG can be stored for at least one year in the dry state without appreciable loss of activity. A hybrid of this method and the succinimydyl active ester has also been developed. PEG activated with p-nitrophenylchloroformate is coupled to the free terminal amino group of nor-leucine, and then the carboxyl group of n-leucine is activated as a succinimydyl ester.[39]

Many additional methods exist, some of which are only used by a limited group of workers or for restricted applications and, hence, these are difficult to evaluate. *The PEG-succinate mixed anhydride method* (Figure 6) has been used to couple PEG to allergens such as honey-bee venom, ovalbumin, and ragweed pollen extract.[29,30,115] However, this activated polymer has a major drawback because it has two sites susceptible to nucleophilic attack and, although one of the sites leads to PEG-modified protein, the second site will give an ester-modified protein (see Figure 6). *The poly(PEG-maleic acid-anhydride)* has been used to attach eight molecules of PEG to a single lysine residue in cholesterol oxidase. Although attachment of eight molecules of PEG per lysine might be useful, the major drawback of this method is the simultaneous introduction of 15 carboxylic acid residues as part of the activated PEG molecules with the subsequent alteration of the charge (Figure 7).[169]

Recently, a new strategy has been applied to link PEG to thiol groups in the protein.[182] This method uses a *PEG-maleimide* produced by reaction of PEG-amine with *N*-maleimido-6-amino-caproyl ester of 1-hydroxy-2-nitro-4-benzene sulfonic acid (Figure 8). Although it has been applied to produce biologically active PEG-cys_3-rIL-2 (a mutant IL-2 with $threonine_3$ being replaced by cysteine), its use will be limited to proteins carrying cysteine residues not involved in sulfydryl bridges or to mutant proteins, which conserve the biological activity after the introduction/replacement of the cysteine residue.

One concern shared by all the above methods is that the coupling step leaves part of the coupling moiety attached to the PEG-protein. This is potentially deleterious in two ways. Firstly, as mentioned above it can serve as an antigenic determinant. Secondly, although the toxicology of PEG has been well studied and is low (see above), some coupling moieties may be toxic. In addition, most methods form relatively toxic coproducts and the PEG proteins thus have to be extensively purified before use. The latter not only jeopardizes some labile proteins but makes testing new product in *in vitro* systems time consuming and costly.

With these problems in mind we explored the use of tresyl chloride as an agent with which to activate PEG for coupling to proteins (Figure 9). Nilsson

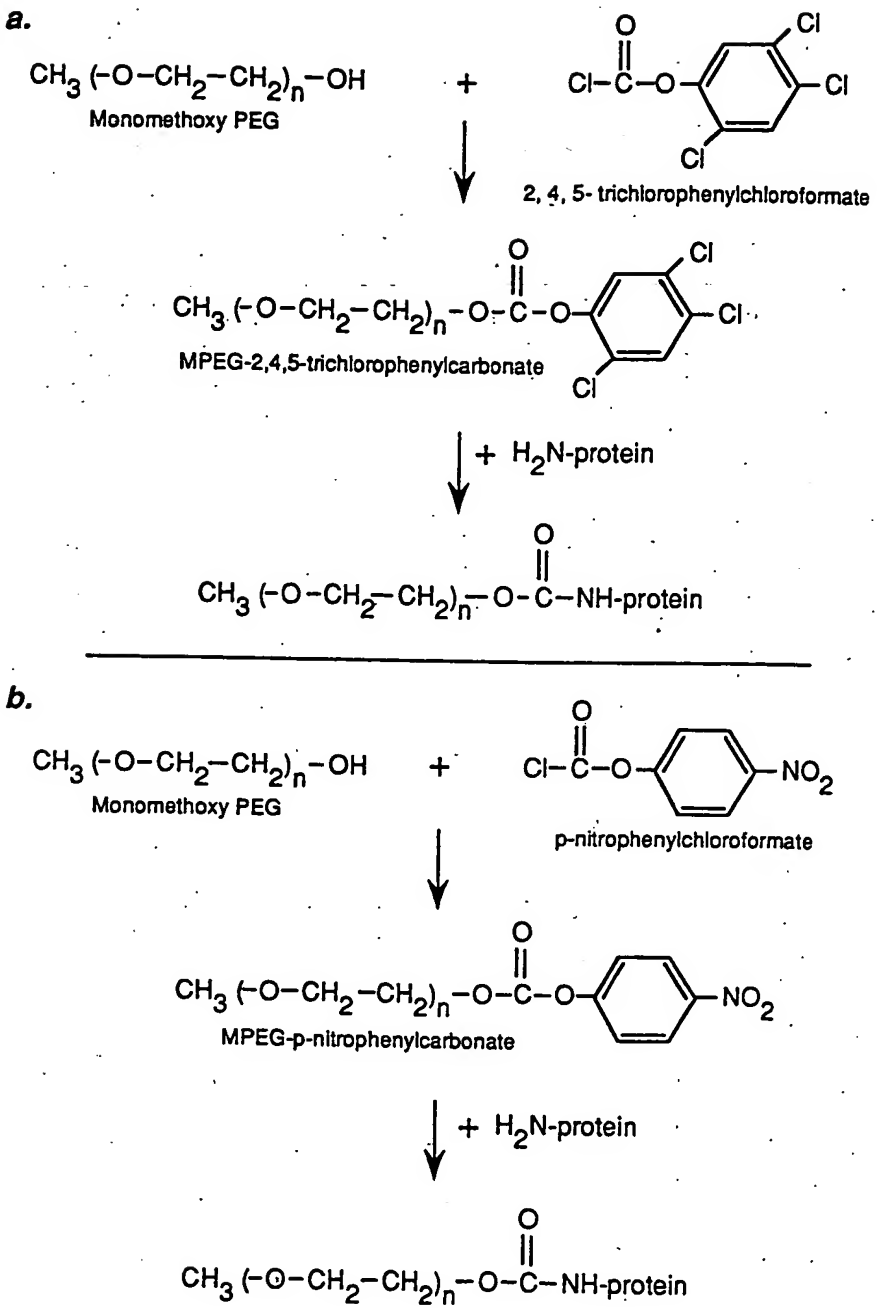

FIGURE 5. Activation of MPEG by phenylchloroformates and reaction of the phenylcarbonate derivatives with amino groups. a) MPEG 2,4,5-trichlorophenylcarbonate; b) MPEG-p-nitrophenylcarbonate (adapted from Veronese et al.[50]).

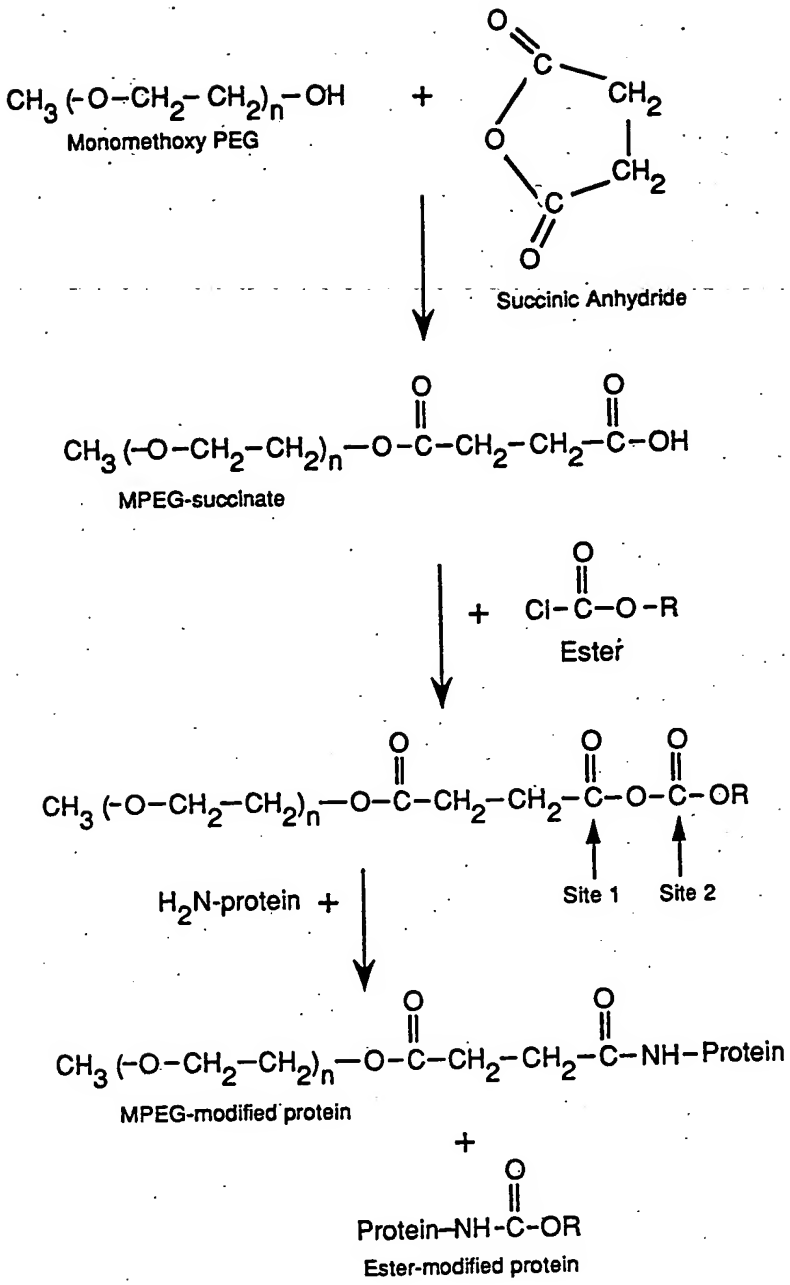

FIGURE 6. Mixed anhydride method for the activation of MPEG and subsequent coupling to a protein.

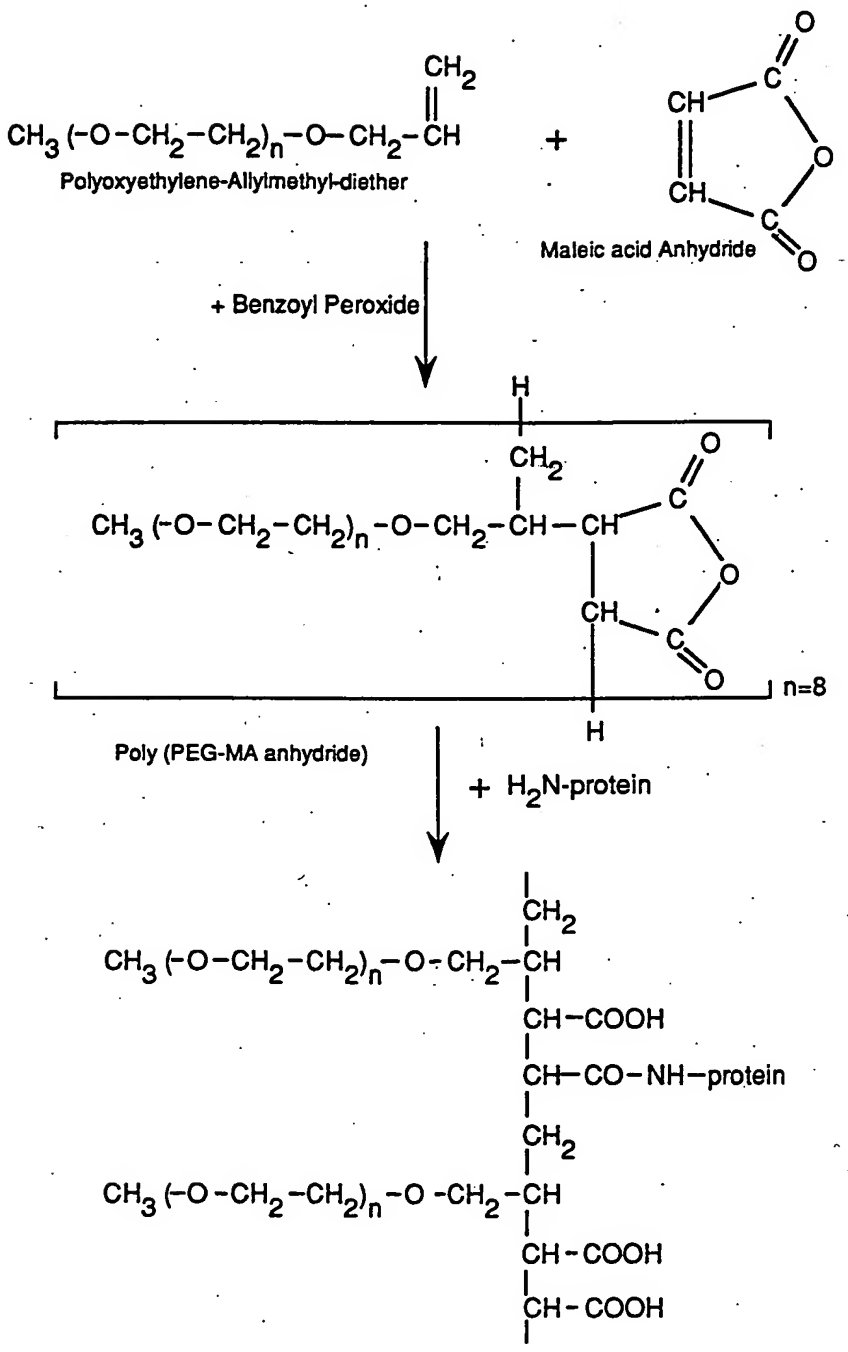

FIGURE 7. Activation of polyoxyetheleneallylmethyldiether with maleic acid anhydride and subsequent coupling to a protein (adapted from Yoshimoto et al.[169]).

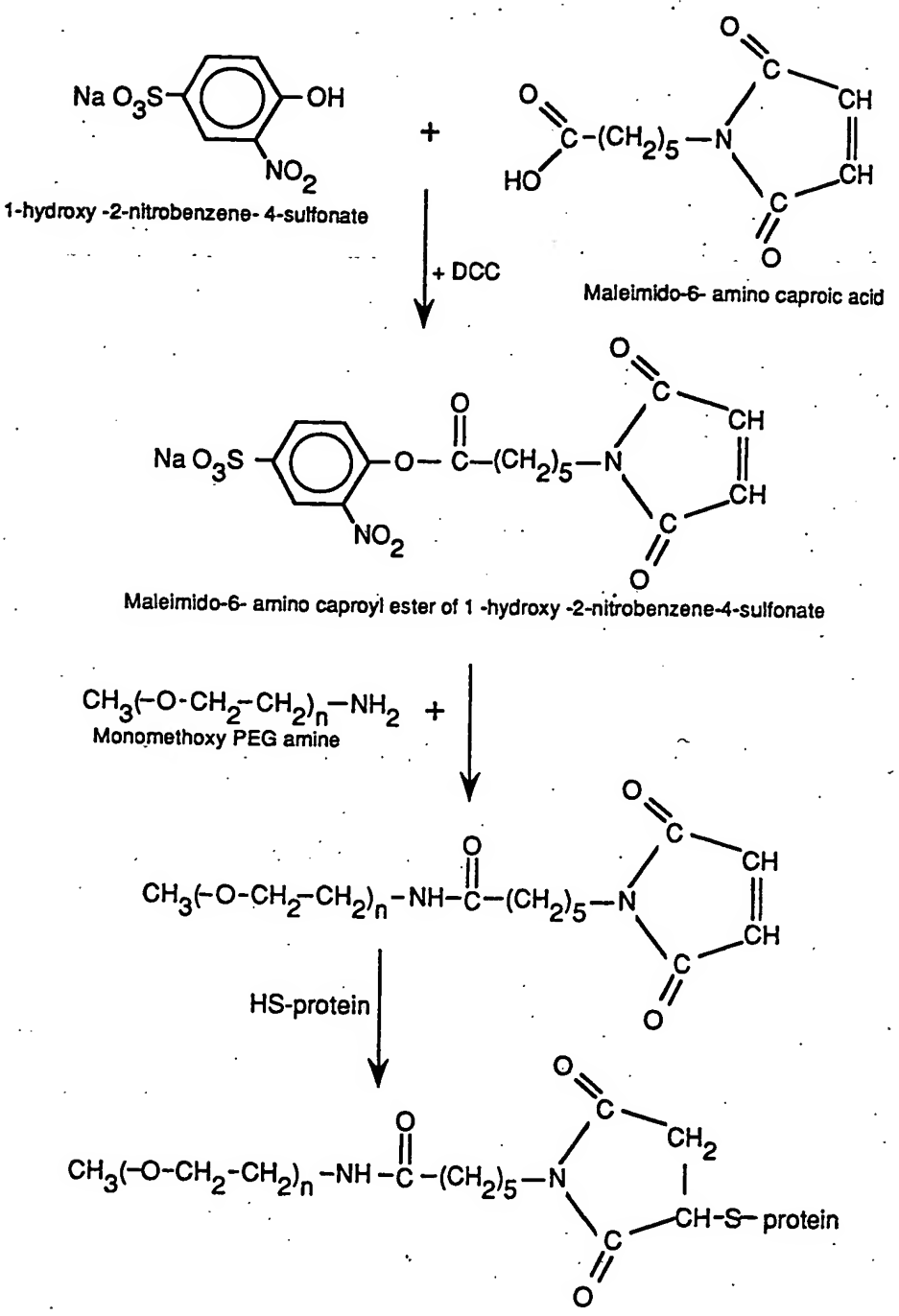

FIGURE 8. Activation of MPEG-amine with *N*-maleimido-6-amino-caproyl ester of 1-hydroxy-2-nitro-4-benzene sulfonic acid and subsequent coupling to a protein (adapted from Aldwin and Nitecki[212]).

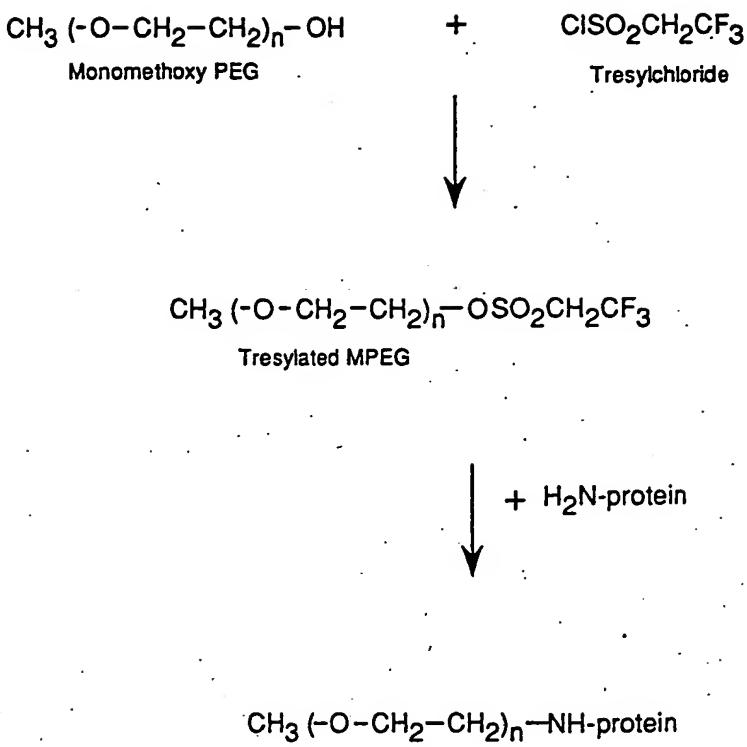

FIGURE 9. Activation of MPEG with tresyl chloride and subsequent attachment to a protein.

and Mosbach had used tresyl chloride (2,2,2-trifluoroethanesulphonyl chloride) to activate agarose and other solid supports carrying hydroxyl groups so that they may be coupled to proteins.[183] We have successfully applied this approach to the activation of monomethoxyPEG (MPEG)[184] and have used the *tresylated MPEG* for coupling PEG to albumin,[184] antibodies,[185] transferrin,[140] and GM-CSF.[186] One attraction of this method is that coupling to proteins takes place quickly and under very mild conditions (e.g. pH 7.5 phosphate buffer, at room temperature)[184] and this could be important for some proteins (for instance, hemoglobin requires a relative low pH in order to keep the salt bridges between some aminoacids, see above). A further advantage over previous techniques is that the reaction mixture is relatively innocuous and does not have to be removed before the PEG-protein is exposed to the target cells in many assay systems[47,187] a feature which greatly facilitates *in vitro* studies of the product. It is unusual among PEG-coupling methods in that only PEG is attached to the protein through a highly stable amine linkage (Figure 9).

PEG-aldehyde (Figure 10) also couples PEG to the protein via a highly stable amine linkage. Several PEG-aldehydes have been prepared.[37,188] PEG-acetaldehyde shares with tresylated PEG that only PEG is attached to the protein (Figure 10).

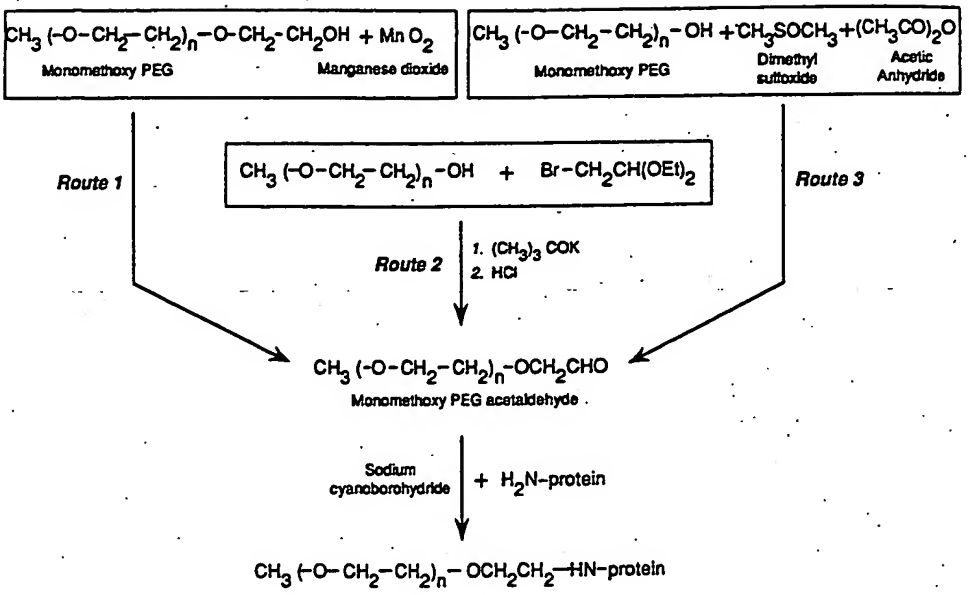

FIGURE 10. Production of PEG-aldehyde and coupling to a protein (adapted from Harris[176] and Harris et al.[175]).

V. ANALYTICAL AND PREPARATIVE PROBLEMS

A. Characterizing the Product

1. Molecular Weight/Size Determination

Covalent attachment of PEG to proteins naturally increases their molecular weight. However, the random coil structure of the polymer seems to produce a much bigger increase in the hydrodynamic radius of the protein than in molecular mass, thus complicating the interpretation of the outcome from electrophoresis, gel filtration etc.

Electrophoretic studies on native proteins using the discontinuous buffer system of Davis[189] have shown reduced electrophoretic mobility (in some instances negligible) in a number of cases including bovine serum albumin,[35] phenylalanine-ammonia-lyase,[19] SOD[12] and α_2-macroglobulin-trypsin complexes.[12] Such a behavior could represent an increase in molecular weight but this has to be enormous to counteract the effect of the increased overall negative charge of the protein (by reduction in the positively charged free amino groups) which is then expected to migrate faster to the anode. Entanglement of PEG chains with the polyacrylamide gel, thus impairing the movement of the protein, was the immediate explanation. However, when thin-layer-electrophoresis of phenylalanine ammonia-lyase was carried out using Sephadex G-75 as the anticonvection medium similar reductions in mobility were observed.[19] Since phenylalanine ammonia-lyase in both unmodified and PEG-modified forms are not held on columns of

Sephadex G-75, but elute in the void volume, it seems unlikely that the reduced electrophoretic mobility in the latter case was due to entanglement of the PEG chains with the solid support.

SDS-PAGE has also been used and, again, the mobility of the PEG-modified proteins was much slower than that expected for the increase in molecular weight.[33,38,158,190] Some of this effect could be due to free PEG, which is known to influence the mobility of SDS-peptides as a function of their molecular weight when added to the electrophoretic solution.[191] With IL-2, attachment of PEG-350 did not alter its electrophoretic mobility although with PEGs in the range 4000 to 20,000 the reduction in electrophoretic mobilities is more pronounced for bigger PEGs.[15]

In gel filtration, a general displacement of the protein profile towards faster elution volumes has occurred upon PEG-modification[10,15,38,43,50,179,192] The molecular size of the PEG-modified adducts calculated from globular protein standards are in excess of the molecular weight expected from the number of PEG chains attached.[10,15] Two observations explain the discrepancy. Firstly, the semilogarithmic plot of the actual molecular weight (calculated as a sum of the contributions of protein and PEG chains) of PEG-hemoglobin conjugates vs. elution volume was not the same as that of globular proteins; and secondly, such a relationship was dependent on the molecular weight of the PEG, with smaller PEGs producing bigger excess of the molecular size than larger PEGs (PEGs within the range 750 to 4000 Mw).[10] Note, however, that the effective molecular size of the conjugates showed a good correlation with the plasma half-life of PEG-IL-2 (up to 70,000 Da)[15] and PEG-hemoglobins.[10] These observations support the validity of the molecular size of the PEG-proteins as measured by gel filtration.

Sucrose density gradients were found to be of no use to estimate the molecular size of PEG-modified phenylalanine ammonia-lyase since the sedimentation rate of the protein decreased as the modification progressed. This anomalous behavior was attributed to alteration in the hydrodynamic properties of the enzyme after covalent attachment of PEG.[19]

2. Charge-Related Techniques

As has been mentioned, electrophoresis in polyacrylamide gels of native PEG-proteins results in reduced mobility, which is not commensurate with the increase in overall negative charge.[12,19,35] However, isoelectric focusing of PEG-modified superoxide dismutase[190] and IL-2[15,33] showed a reduction in pI as expected from the reduction in positively charged amino groups. Furthermore, as more PEG molecules were attached to IL-2, the proportion of species with lower pI values increased.[33]

PEG-modified proteins showed a reduced electrostatic interaction with both cation- and anion-exchange supports.[35,178,190] The attachment of PEG to amino

groups should reduce the number of positive charges (cation number) in the protein and thus a reduced interaction with a cation-exchanger is not unexpected. However, although SOD binds to a weak cation exchange column at pH 5 at relatively high ionic strength (0.02 M sodium acetate), PEG-SOD only binds to the cation-exchanger at low ionic strength (0.001 M sodium acetate). Part of this reduced binding has been attributed to the differences in pI between SOD and PEG-SOD; the pH at which the chromatography is performed is below the pI of SOD and above the pI of PEG-SOD and thus favors the positively charged form for SOD and a more neutral form of PEG-SOD.[190] Additionally, steric shielding might make some contribution.

In anion-exchange chromatography several features have been observed. To bind comparable amounts of PEG-ovalbumin and ovalbumin to a DEAE ion-exchanger, columns at least 100 times larger were needed for the former and, again, low ionic strength buffers were needed.[178] These requirements may explain the failure to bind PEG-albumin to anion-exchangers.[35] In contrast, PEG-SOD binds to anion-exchangers under the same conditions as SOD and the retention times of the two species are not markedly different.[190] However, although SOD elutes as a sharp peak, PEG-SOD shows a very broad elution pattern. This has been attributed to either interference of the PEG chains with the ion-exchange process or PEG chains leading to a mixed mechanism retention, which could produce band tailing.[190]

3. Hydrophobic Interaction Chromatography and Allied Techniques

Both hydrophobic interaction chromatography and reverse-phase chromatography have shown an increased hydrophobicity for several PEG-proteins when compared with the unmodified counterparts.

Using a TSK-phenyl-5-PW column, PEG-IL-2 conjugates eluted after the unmodified IL-2 but the elution volume for all the conjugates was the same regardless of the stoichiometry of the modification reaction.[33] In contrast, PEG-ovalbumin conjugates (PEG-OA) with more PEG chains bound more avidly to the column and, thus, there is a relationship between hydrophobicity and average degree of modification.[178] However, it is surprising that for each individual PEG-OA preparation sharp elution peaks are obtained, as though the preparation contained only one defined PEG_n-OA species, which is very unlikely (see below). PEG itself did not bind to the column[33] and this seems to preclude retention of the PEG-modified proteins in the column by the mere interaction of the attached PEG chains with the matrix.

PEG-SOD has a longer retention time than the unmodified SOD when chromatgraphed in a C_4 reverse-phase column. In addition, the sharp elution peak of SOD is replaced by broad bands upon PEG-modification.[190]

B. Measuring the Degree of Substitution

1. Direct Estimates

The average degree of modification can be measured directly by quantifying the number of PEG-chains molecules or indirectly by measuring the number of unmodified amino groups that remain.

The direct quantification of the PEG implies isolation of the PEG-protein conjugates from the reaction mixture containing the unreacted PEG. This has been done mostly by ultrafiltration.[38,133] Quantification of the PEG can be done using a radiolabeled PEG as starting material,[134] but radiolabeled PEGs with only one reacting hydroxyl group (see Section IV) are not commercially available. Production of radiolabeled PEG is feasible but involves several steps[134] that are expensive and time consuming.

Using an activated PEG that leaves a coupling moiety between the PEG and the protein provides a means to quantify the PEG. This strategy has been applied by Veronese et al.[39] and by Yamasaki et al.[181] using nor-leucine as an aminoacid spacer-arm (see Section IV). The amount of polymer bound is calculated from the nor-leucine content by aminoacid analysis after acid hydrolysis.[39,181]

A second approach to quantify PEG takes advantage of the single, sharp signal at $\delta = 3.7$ ppm in ^1H NMR. It is claimed that the concentration of PEG in the conjugates can be calculated to within 2% by comparison with appropriate standards.[178] ^{13}C NMR has also been used, and by comparison of peak areas of carbonyl carbons of the hemoglobin with methylene carbons of the PEG the latter is quantified.[10] A final strategy is to measure the net weight after freeze-drying the PEG-modified protein solution of a known concentration.[10] This approach, however, relies on the assumption of the PEG being homogeneous in molecular weight, which is very unlikely.

The estimation of amino groups can be done by the colorimetric method described by Habeed[193] or by a fluorometric method using fluorescamine.[194] Both methods rely on an accurate determination of the protein concentration in both unmodified and PEG-modified preparations. The colorimetric method is easy to perform but requires protein solutions with concentrations of the order of milligram per milliliter. The main disadvantage is that the unreacted PEG has to be removed prior to assay and that the reagent can react with other nucleophiles such as cysteine sulphydryl groups.[194] The fluorescamine method provides reproducible results in the presence of unreacted PEG and thus can be performed directly over the reaction mixture. A second advantage over the colorimetric method is the sensitivity because amino groups can be detected in as little as 50 ng of protein.[195]

The unmodified lysine residues relative to the total in the protein can also be estimated by comparison of the aminoacid compositions of PEG-modified and unmodified proteins evaluated by a standard aminoacid analyzer.[196] However, the sensitivity of this technique will vary largely from protein to protein since it depends on the relative number of lysine vs. total aminoacids in the protein and

the degree of modification. In addition, this method will be limited to PEG-protein conjugates where the covalently bonded PEG is not removed by the acid hydrolysis required in the analysis. A direct bond of PEG to the protein as provided by tresylated PEG seems to be the most resistant to hydrolysis.

Phase partitioning in aqueous two-phases of PEG and dextran provides an additional parameter, the partition coefficient (ratio of protein concentrations in top and bottom phases), which is related to the degree of modification.[184] The attachment of PEG chains to the protein increases its partition into the PEG-rich top phase of the systems. The linear relationship maintained between log partition coefficient and the number of PEG molecules bound[184] makes this system extremely sensitive to measure PEG-modification because the partition coefficient has a unique value for each PEG_n-protein conjugate.

2. Indirect Estimates

Determination of the molecular weight of the PEG-protein conjugates is a very attractive method for measuring the degree of modification. However, since discrepancies between molecular size and molecular weight are generally found for PEG-proteins (see above), care should be taken when either electrophoretic or chromatographic techniques are used to estimate the degree of substitution of a preparation. In addition, analysis of PEG-modified IL-2 preparations by size exclusion chromatography and SDS-PAGE resulted in the former providing larger molecular weights,[15] whereas for PEG-SOD size exclusion chromatography provided a smaller molecular weight than SDS-PAGE.[190] These contrasting results add another limitation to the use of either technique to estimate the degree of modification.

The isoelectric point of the PEG-protein conjugate should also provide an indication of the degree of modification because with the covalent attachment of each PEG molecule one positive charge (of the primary amino group) is removed. This principle has been exploited for PEG-IL-2 but the resolution of the technique seems to vary for PEGs of different molecular weights.[33]

C. Heterogeneity and Fractionation in Individual PEG-Protein Adducts

In general, proteins have more than one residue to which PEG can attach and this will impose the production of a statistical mixture of PEG_n-protein adducts under all reaction conditions (except where complete substitution of the available residues is achieved). Increasing the molar ratio of activated PEG to protein will increase the average substitution and will change the distribution of the PEG_n-protein conjugates but will not allow the production of individual conjugates with an intermediate value for n. In addition, the mixture will be heterogeneous not only with respect to the number but also to the location of the PEG molecules

on the protein (unless subtle differences in pKa and accessibilities for individual lysine residues impose an order for the substitution). These problems have rarely been adequately addressed and for some products may be very important. For example, with PEG-GM-CSF the enhancement of neutrophil priming activity is related to the number of PEG molecules attached.[47] With PEG-ovalbumin and PEG-uricase there is an optimum number of PEG molecules attached to produce the most effective tolerance (above it, the PEG-conjugates are less effective).[114,197] On the other hand, since PEG-modification conveys to the protein both beneficial and detrimental effects, it is desirable to establish the number of PEG-protein species present in a sample and the contribution of each individual conjugate to the new properties. This will allow selection of the best PEG-protein adduct for each particular application.

Only a few attempts have been made to reveal heterogeneity and none of the analytical techniques used emerges as uniquely tailored to resolve any PEG-protein preparation into individual components. Two main problems have been encountered. First, the number of species in a preparation varies depending on the technique used; furthermore, the resolution of the different analytical techniques varies between proteins. This has been documented for PEG-IL-2 and PEG-SOD preparations. Isoelectrofocusing reveals more heterogeneity for PEG-IL-2 conjugates (prepared with PEGs of different molecular weights) than for SDS-PAGE.[15,33] In contrast, PEG-SOD is resolved into six bands by SDS-PAGE and only three by isoelectrofocusing.[190]

The second problem is the lack of resolution in individual components even though heterogeneity is revealed. This is the case with most chromatographic techniques (e.g., gel filtration, ion-exchange, and reverse-phase) where, in general, very broad profiles are produced.[15,190,198] An additional problem with gel filtration is the effect of contaminating PEG on the elution profile.[199,200] Larger elution volumes of proteins in gel filtration in the presence of PEG were explained in terms of the unfavorable interactions between PEG and both the proteins and the gel matrix.[200] Such interactions decrease with the protein molecules being in the cavity of the gel matrix, thus leading to the slower elution.[200] New solid supports such as PEG-silica[201,202] may provide a new avenue to analyze and fractionate PEG-protein preparations.

Phase partitioning with the counter-current distribution procedure has shown heterogeneity for PEG-BSA preparations[184] and for PEG-GM-CSF[213] but resolution into individual conjugates has not been observed in any case.

It seems likely that at least a double-dimension analysis will be required. We have demonstrated that within unresolved FPLC profiles for PEG-GM-CSF preparations, the faster eluting fractions (excluding the fastest and only clear peak) contained species more PEGylated than the slower eluting fractions. This is unequivocally shown by the decreasing value for the partition coefficient in aqueous two-phase systems from fast to slow eluting fractions. In addition, by applying mathematical modeling to test different assumptions about the elution and partitioning behavior, it has been possible to construct mathematical solutions to the composition of PEG_n-GM-CSF mixtures and, hence, to identify fractions

having an individual conjugate with respect to the number of PEG molecules attached with a purity above 95%.[203]

A final potential problem is that in all the partially resolved preparations the number of components is below the maximum possible, and it is very unlikely that any of the resolved species correspond to a defined PEG_n-protein conjugate. To address this issue, peptide mapping of fractionated preparations homogeneous with respect to the number of PEG molecules attached will be required to identify the position of the lysine residues involved.

VI. CONCLUSIONS

PEG-modification of proteins represents a significant advance in protein pharmaceuticals. The generic benefits of PEG-modification (improved half-life and bioavailability, resistance to proteolysis, and reduced immunogenicity and antigenicity) and the low toxicity of PEG make this an attractive avenue. PEG-proteins with increased bioavailability are likely to have more reasonable dosing schedules (the actual scheduling will need to be evaluated empirically because the prolonged lifetime of PEG-proteins would produce different plasma concentration profiles for the modified and unmodified proteins whatever schedules are used, i.e., the peak concentrations and the minimum concentration achieved between doses would vary).

The induction of tolerance by PEG-protein conjugates opens up the possibility of using PEG-conjugates of different xenogeneic, biologically active proteins as tolerogens for the development of therapeutic regimens requiring the administration of the unmodified proteins. This will be especially important for proteins where lack of immunogenicity/antigenicity is compromised with a substantial loss of biological activity.

A second important application of PEG-protein is in biotechnology. The conserved biological activity of PEG-proteins in organic solvents will allow synthesis of compounds with a 3D structure determined by the conformation of the active site of the enzyme (and this is unlikely to be obtained by classical chemical synthesis). PEG-proteins will also have a role in biotechnological applications of aqueous two-phase systems.

Dissociation of biological activities will lead to production of proteins with a different spectrum of applications and unexpected mechanisms of action. In addition, attachment of PEG to specific sequences on the protein might contribute to elucidate its role in the biological function (activity, clearance, inactivation, etc.). The new specificities (both substrates and reactions) found for PEG-enzymes in organic solvents should provide, by comparison to enzymes having that biological activity in their native state, new information on the domains of the protein involved in the catalysis and the shape of the catalytic pocket.

The further development of the coupling methods to produce PEG-proteins uncontaminated with unreacted active PEG and coproduct, together with frac-

tionation procedures leading to individual PEG_n-protein conjugates, should ensure a rapid advance in the use of PEG-proteins for clinical and biotechnological applications.

ACKNOWLEDGMENT

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